

THE PHYSICOCHEMICAL PROPERTIES
AND BIOLOGIC ACTIVITIES OF
 γ M-GLOBULINS

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INTRODUCTION

This dissertation describes a series of investigations into the nature of the reductive depolymerization of horse anti-pneumococcal polysaccharide (Type I) γ M-globulin, and reaggregation of the subunits by reoxidation of disulfide bonds. Several preliminary observations on the activity of horse anti-pneumococcal antibodies have appeared in a previous publication by the author (Hill, 1964). These observations may be summarized as follows:

- 1) The γ M-antibody at a concentration of 0.5% had an $s_{20,w}$ of 17.3S and the reductively produced subunit at the same concentration had an $s_{20,w}$ of 6.1S.
- 2) Concentrations of 2-mercaptoethanol of at least 0.075 M were required to depolymerize completely the γ M-antibody.
- 3) Depolymerization under these conditions followed by treatment with 1 M propionic acid resulted in the liberation of 32% of the light chains of the molecule.
- 4) γ M-globulin contains some determinants that are antigenically different from some determinants on γ G-globulin.
- 5) A portion of the anti-pneumococcal antibodies could be specifically precipitated with a polygalacturonic acid (pectic acid). This is a reflection of the high galacturonic acid content of the Type I polysaccharide.

Many of the physicochemical properties of the specific γ M-antibody paralleled those found by others for various pathologic macroglobulins. Since relatively large amounts of specific γ M-antibody were available for physicochemical studies, a detailed study of its molecular properties was undertaken in conjunction with measurements of its various biologic activities.

Previously, with a few exceptions, it had been assumed that reduction with mercaptoethanol somehow altered the molecule in such a way that it no longer reacted with its antigen. A detailed study of the effect of reduction on the biologic activity of γ M-antibody would require a knowledge of the mildest conditions necessary to achieve depolymerization, the extent of depolymerization at reducing agent concentrations lower than necessary to depolymerize all the molecules, and the number of disulfide bonds that are broken during the course of depolymerization of the molecule.

Once the questions regarding the physicochemical manipulations of the γ M-antibody were answered, it became necessary to develop or adapt techniques to detect and quantitate the antibody activity of the intact γ M-antibody as well as any activity retained by the subunit. Visualization of any antibody binding activity by the subunit would depend on indirect precipitation of the antigen which had been labelled in a manner permitting its easy detection in a milieu of proteins. The conditions of labelling had to be such that the antigenic determinants of the antigen, the SI polysaccharide in our case, were not destroyed. To this end, it was concluded that the most desirable label would be a radioisotope which could be used for

qualitative detection of SI precipitation, by the technique of radio-immunoelectrophoresis, and which could also be used for quantitative determinations of its binding to γ M-antibody and its subunits.

Since it has been established that depolymerization of γ M-antibody abolishes or diminishes secondary effects of its antibody activity, the question that presents itself is: Can at least some of these activities of macroglobulin be restored if one uses techniques of oxidative reassembly that are known to restore activity to certain enzymes which are also inactivated reductively?

The experiments presented in the Results section of this dissertation in part help to answer these questions. As the author delved further into the nature of γ M-antibody, more questions were raised, and some of these unanswered questions are treated in the Discussion section.

REVIEW OF LITERATURE

Immunology had its beginning in 1798 when a country doctor, Edward Jenner, observed that dairy workers did not contract smallpox if they had previously had a rather innocuous disease called cowpox. Jenner deliberately infected persons with cowpox thereby successfully protecting them from smallpox. From this momentous beginning immunology has grown in two directions. The first is a continuation of the work Jenner began, that is, protecting people from the ravages of disease. The second direction involves a continuing attempt to understand the basic mechanisms of the immune response. The attempt to quantitate antigen-antibody reactions and to characterize the molecules involved in immune reactions has led to the development of a new discipline, immunochemistry. Immunochemistry employs the systems and nomenclature of immunology and the tools and methods of analytical biochemistry. The concerns of immunochemists have expanded beyond the field of immunology and they have obtained information about the structure and properties of certain proteins and other macromolecules that has contributed to biochemical knowledge.

By 1936 it had been clearly established that antibodies were actually modified serum proteins. Techniques had been developed that allowed one to obtain solutions in which 98% or more of the protein was specific antibody. Heidelberger and Pedersen (1937)

were able to study the sedimentation characteristics of such purified antibodies derived from different species and reactive with different immunogens. The antibodies studied by these authors were isolated from rabbits immunized with either egg albumin or Type III pneumococci and horses immunized with Type I pneumococci. The antibodies from the rabbit reactive with either immunogen were found to have a sedimentation coefficient (s_{20}) of 7.0S to 7.4S while anti-pneumococcus antibodies from the horse had an s_{20} of 18.2S. In an extension of this work, Kabat and Pedersen (1938) determined the molecular weight of antibodies from several animal species reactive with the same pneumococcal polysaccharide. Their results may be summarized as follows: 1) Antibodies from rabbit and monkey were found to have an $s_{20} = 7S$ and a molecular weight of about 157,000 and 2) antibodies from pig, cow, and horse were found to have an $s_{20} = 18S$ and a molecular weight of 930,000. From these data the authors concluded that mammals fell into two groups with respect to the size of antibody molecules which they synthesized. One group was composed of those animals that produced antibodies of a size similar to that of the globulins found in largest amount in human serum, i.e. having an $s_{20} = 7S$ while the other group consisted of animals producing antibodies of a much larger size. It was later shown that while the response of the horse to pneumococcal polysaccharides was consistent with this grouping, the reaction of the horse to diphtheria toxoid, as well as many other immunogens, was inconsistent. Immunization of the horse with many immunogens could and did lead to the production of antibodies of the smaller ($s_{20} = 7S$) variety (Pappenheimer, et al., 1940).

The molecules in the group of serum proteins known as antibodies are formed in response to a stimulation by a 'foreign' substance, called an antigen or an immunogen, and each antibody molecule reacts specifically with that immunogen, or a portion thereof, which initiated its formation. Antibodies have the physicochemical characteristics that place them in the group of serum proteins that have been called γ -globulins. The γ -globulins were operationally defined, in part, as that group of serum proteins that migrated slowest in an electrical field when in an alkaline solution, i.e. pH 8.6. Closer scrutiny has indicated that antibody activity can be found in almost all of the electrophoretic groups of serum proteins. It has been suggested that the term 'immunoglobulin' be adopted which more accurately defines the groups than does ' γ -globulin'. The serum immunoglobulins are grouped into one of three distinct classes, γ G-, γ A-, or γ M-immunoglobulins (Kunkel, 1960), called alternately IgG-, IgA-, and IgM-immunoglobulins respectively (Bull. WHO, 1964). A fourth class of immunoglobulins, γ D-immunoglobulin, has been reported, but its function is as yet unknown (Rowe and Fahey, 1965). The above system of nomenclature reflects the close interrelationship of the immunoglobulins. Molecules of the three principal classes differ markedly from each other in their physicochemical properties and in their antigenic constitution. However, they also have in common certain peptide sequences and polypeptide chains (Fahey, 1963; Migita and Putnam, 1963).

Those pathologic proteins produced by multiple myeloma patients have served as a source of globulins that are more homogeneous than

normal γ -globulin. The myeloma protein of a multiple myeloma patient seems to be produced by a clone of cells arising from a single stem cell and the pathologic protein may comprise up to 80% of the patient's total serum proteins (Putnam, 1960).

A large fraction of the multiple myeloma patients excrete a protein, called Bence-Jones protein, in their urine. It has been conclusively demonstrated that these are monomers or dimers of light chains of the same antigenic type as those found on the intact myeloma protein of the same patient (Heremans, 1960).

Of all the immunoglobulins, the γ G-globulins have been most thoroughly studied. This is mainly because of their relative abundance in normal serum and the ease of their isolation as a homogeneous preparation. A molecule of γ G-globulin has been found to consist of two pairs of polypeptide chains covalently joined together through disulfide bonds. The pairs of polypeptide chains consist of one light chain, which has a molecular weight of 22,000 to 23,000 (Pain, 1963; Small, *et al.*, 1963; Marler, *et al.*, 1964; Small and Lamm, 1966), and of one heavy chain, having a molecular weight of about 50,000. The total molecular weight of γ G-globulin is 140,000 to 150,000 (Pain, 1963; Small, *et al.*, 1963; Marler, *et al.*, 1964; Small and Lamm, 1966), and the molecule has a sedimentation coefficient of 6.5S. The heavy chain carries the antigenic determinants that are unique to the class of γ G-immunoglobulins and it is referred to as the γ -chain. The glycopeptide moiety, which accounts for 2% to 3% of the total weight of the γ G-molecule, is also associated with the γ -chains (Fleishman, *et al.*, 1963; Press and Porter, 1964). The light chains of the human species occur as two major antigenically

distinct types and are denoted as K- (Kappa) and λ - (Lambda) chains. Light chains are also found on γ A- and γ M-globulins and seem to be essentially similar regardless of the class of globulins from which they are isolated (Chaplin, et al., 1965; Wier, 1964). The γ G-immunoglobulins have been found to contain two antigen binding sites per 140,000 molecular weight (Karush, 1956, 1957; Singer and Campbell, 1955).

The information available on γ A-globulins is relatively incomplete. These immunoglobulins are present in human serum in lower concentrations than γ G-globulins. Estimates of γ A-globulin concentration have ranged from about 150 mg% (Heremans, 1960; Chodirker and Tomasi, 1963) to 400 mg% (Fahey and Lawrence, 1963); however, these proteins are found in relatively high concentration in various human secretions, i.e. those of the lactating mammary gland, intestinal secretions, and nasal secretions, saliva and tears (Hanson, 1961; Tomasi and Zigelbaum, 1963; Chodirker and Tomasi, 1963; Tomasi, et al., 1965). A γ A-antibody has been isolated from precipitates, formed with horse antiserum, along with other immunoglobulins; however, its ability to precipitate antigen is in doubt (Klinman, et al., 1965). The γ A-globulins appear to contain heavy chains of higher molecular weight than that of γ G-globulin (Cebra and Small, 1966). These heavy chains are immunogenically distinct and are referred to as α chains. The γ A-globulin molecules also contain light chains, as do the γ G- and γ M-globulins (Wier, 1964). γ A-globulins have an $s_{20,w} = 7S$ to $10S$ (Smith and Brown, 1950) but may occur as multiples of the 7S molecule. The glycopeptide moiety

present in human and horse γ A-globulin comprises some 5% to 10% of the total weight of these proteins (Heremans, 1959; Schultze, 1959).

The remaining class of immunoglobulins, the γ M-globulins, are presently coming under intensive study and it is this class of immunoglobulins that is the subject of this dissertation. Much of the information about γ M-globulin that has accumulated to date has come from investigations of those γ M-globulins produced in large quantities by humans suffering from Waldenström's macroglobulinemia (Waldenström, 1944; Kunkel, 1960). The macroglobulin from these patients has been found to have an $s_{20,w}^0 = 18S$ and a molecular weight estimated to be about 1,000,000. The molecule has a somewhat higher carbohydrate content than does γ G-globulin. Normal human γ G-globulin was found to contain 2.49% to 2.6% carbohydrate while γ M-globulin contained 9.8% to 10.23% carbohydrate (Miller-Eberhard, Kunkel and Franklin, 1956; Miller and Metzger, 1965). The antibodies produced by the horse, cow, etc. in response to pneumococcal polysaccharides, as mentioned above, are of the γ M-globulin class. This γ M-antibody response was found not to be restricted to the few animals noted above but to be a rather ubiquitous response. Bellanti, et al. (1963) showed that the earliest detectable antibody produced by rabbits and humans upon immunization with various Salmonella species was of the γ M-globulin type. Other examples of γ M-globulin production include: Human isoagglutinins (Pedersen, 1945), rheumatoid factor of man (Edelman, et al., 1958; Heimer, et al., 1958), and rabbit anti-sheep red blood cell antibodies (Fahey and Humphrey, 1962). More recently, γ M-antibodies have been isolated which were specifically reactive with one of two haptens.

Rockey, et al. (1964) isolated γ M-antibodies to the hapten p-azophenyl- β -lactoside from immune horse serum. Onoue, et al. (1965) isolated γ M-antibodies from the rabbit reactive with the hapten p-azobenzenearsonate. The titer of circulating γ M-antibodies is significantly higher than the γ G-antibody only early in an immune response, a notable exception being the case of the pig, cow, or horse immunized with the various pneumococci.

Testing the assumption that the macromolecular globulin from macroglobulinemic patients was an aggregate of smaller molecules, Deutsch and Morton (1957, 1958) attempted to dissociate the molecules using various combinations of salt, pH and temperature, but the native molecules retained their integrity during these manipulations. However, addition of sulfhydryl compounds such as 2-mercaptoethanol or cysteine resulted in the conversion of the macroglobulins (18S) to subunits (6.5S). The conditions of reduction used by Deutsch and Morton were 0.1 M mercaptan in phosphate buffer ($\mu = 0.2$; pH = 7.4) and incubation at room temperature for 24 to 48 hours. If the mercaptan was subsequently removed by dialysis the amount of 6.5S material was decreased and there was an appearance of components which sedimented faster (13S and 18S). Removal of the mercaptan by dialysis in the presence of 0.02 M iodoacetamide, a sulfhydryl blocking agent, prevented the reassociation and all the protein remained as the subunit. Dissociation of γ M-antibody under the conditions described above led to a concomitant loss of antibody activity as measured by precipitation or agglutination. Thus the decrease of antibody activity in serum following reduction

has been used as an indication of the proportion of specific activity associated with the γ M-globulin. Reduction of γ G-antibody under the same conditions does not appear to affect its precipitating or agglutinating activity or its physicochemical properties. Thus mild (0.1 M mercaptan) reduction appears to destroy the ability of γ M-antibody of the appropriate specificity to agglutinate or immobilize bacteria (Bellanti, et al., 1963; Nossal, et al., 1964), to agglutinate erythrocytes (Fudenberg and Kunkel, 1957; Chan and Deutsch, 1960; Bauer and Stavitsky, 1961), to sensitize erythrocytes for complement lysis (Stelos and Taliaferro, 1959), to neutralize phage (Uhr and Finkelstein, 1963; Bauer, et al., 1963) and specifically to precipitate antigen (Josephson, et al., 1962; Benedict, et al., 1963). In only one of these studies was an attempt made to isolate specific macroglobulin antibody and to correlate the apparent loss of activity with reductive depolymerization (Fudenberg and Kunkel, 1957). Reduction of the red blood cell cold agglutinins with sulfhydryl reagents produced 6.5S subunits from the antibody. These subunits were found to be neither capable of agglutinating the red blood cells nor of rendering the red blood cells susceptible to agglutination by various sera in a Coomb's test. However, another of the above studies (Chan and Deutsch, 1960) suggested that there was conservation of antibody combining sites following reduction. 'Rh' saline agglutinin titers fell to zero when dialyzed vs 0.1 M mercaptoethanol. Removal of the mercaptan by dialysis vs buffer did not restore the ability of the serum to agglutinate red blood cells, however, such cells treated initially with the reduced serum could be agglutinated with an antibody to the saline agglutinins. The proteins rendering the

cells Coomb's reagent positive were, most probably, subunits of the agglutinin that had an intact antibody site. In subsequent experiments, Deutsch and Fudenberg, using isolated saline agglutinins, could not demonstrate any Coomb's reagent activity of the reduced antibody (Chan and Deutsch, 1960). More recently Jacot-Guillarmod and Isliker (1962, 1964) have shown that reduced human anti-A and anti-B γ M-globulins could inhibit hemagglutination by intact antibody and could also specifically render the appropriate erythrocytes susceptible to agglutination by an anti-globulin serum. Onoue, et al. (1964) used yet another technique, radioimmuno-electrophoresis (Hochwald, et al., 1961), to demonstrate the binding of radiolabelled antigen by γ M-subunits. The γ M-antibody was isolated from rabbits immunized with p-azobenzene-arsenate conjugates. The antibody was totally reduced to 6S subunits and then electrophoresed in agar gel. The slides were then developed with a mixture of sheep anti-rabbit γ M-globulin and p-¹³¹I azobenzene-arsenate-insulin conjugate. Radioautographs indicated that the labelled antigen was specifically bound in the band of precipitation given by the subunits.

Although the reductively produced subunits of γ M-antibody are incapable of eliciting many of the secondary effects of the antigen-antibody interaction, i.e. those that are due to its cross-linking properties, at least a portion of these activities can be restored by oxidative recombination (Jacot-Guillarmod and Isliker, 1962; Schrohenleher, et al., 1964). Jacot-Guillarmod and Isliker succeeded in restoring agglutination activity of reduced human anti-A or anti-B γ M-isoantibodies by dialyzing the reduction mixture against Veronal.

buffer, pH 7.4, according to the procedure which was used by Deutsch and Morton (1958) to recombine subunits of Waldenström's macroglobulin. Jacot-Guillarmod and Isliker could not demonstrate recombination of the subunits or recovery of activity if the reduction was accomplished using a sulfhydryl reducing agent such as sodium thioglycolate or cysteamine. Reduction with sodium borohydride (0.01 M to 0.05 M) did not completely abolish the activity of the antibody, however, recovery of a majority of the initial activity by reoxidation was demonstrated. Likewise, Schrohenloher, et al. (1964), reoxidized rheumatoid factor using the same method and restored a significant level of its ability to precipitate with aggregated human γ -globulin.

A feature that distinguishes an antibody from other proteins is its ability to combine specifically with antigen. The antigen-antibody interaction is most easily visualized when the two components are in the proper proportions so that a precipitate is formed. If one attempts to explain this precipitation on a molecular basis, it must be postulated that the antigen be capable of having more than one antibody bound to it and conversely, that the antibody must be capable of binding more than one antigenic site. Ample evidence is now available that γ G-antibody contains two and only two antigen binding sites (Karush, 1956, 1957). The number of binding sites of a γ M-antibody molecule has not been established with certainty. Recently, Onoue, et al. (1965) presented evidence that indicates there are six binding sites per 1,000,000 molecular weight for rabbit γ M-anti-p-azobenzeneearsonate. If the recently determined molecular weight for this molecule, of 850,000 to 900,000 (Lamm and Small, 1966)

is accepted then there are more likely five sites per molecule, or a number equivalent to the number of 6S subunits that make up the whole molecule.

The secondary biologic activities of antibodies differ. The more important of these differences may be summarized as follows:

a) Agglutination and its inhibition. Draff and Shulman (1965) found that, on a weight basis, γ M-antibody had a higher agglutinating activity than did γ G-antibody to the same hapten. These authors isolated rabbit γ G- and γ M-antibodies to the phenylarsonate hapten. Then, using human erythrocytes coupled to arsanilate as the indicator, they found that on a weight basis four times as much γ G-antibody as γ M-antibody was required to give the same reproducible end point. Calculations based on the currently accepted molecular weights of γ G- and γ M-globulin indicates that, on a molecular basis, the γ M-antibody is 26 times as effective as γ G-antibody. A second study by these authors determined the least amount of hapten required to inhibit the agglutination of the conjugated erythrocytes by a particular class of antibody. It was found that about 200 times as much hapten was required for inhibition of the γ M-antibody as was needed for inhibition of an equal weight of γ G-antibody.

The results of Robbins, et al. (1965) are in close agreement with the results cited above. These authors investigated the ability of purified rabbit γ G- and γ M-antibodies to Salmonella typhimurium to agglutinate these bacteria. They found that on a molar basis, γ M-antibody was some 22 times more efficient in agglutinating the bacteria than γ G-antibody. The other parameters which were also investigated will be discussed below.

A more striking difference between the ability of γ G- and γ M-antibody to cause agglutination was shown by Greenbury, et al. (1963). γ M- and γ G-antibodies were isolated from rabbits immune to human blood group A erythrocytes and their ability to cause hemagglutination was compared using a '50% agglutination' end point. They found that while only 25 molecules of γ M-antibody per cell were required to produce this end point, 19,500 molecules of γ G-antibody per cell were required to achieve the same effect.

b) Opsonization. The in vivo clearance of S. typhimurium was used by Robbins, et al. (1965) to quantitate the opsonic activity of γ G- and γ M-antibodies to this organism. These authors found that pretreatment of the bacteria with as little as 0.0006 mg/ml of γ M-antibody would result in phagocytosis of 50% of the organisms within one hour after their injection into the tail vein of a mouse, while concentrations of 0.131 mg of γ G-antibody per ml were required to produce the same degree of phagocytosis. It is interesting to note that reduction of the γ M-antibody with 2-mercaptoethanol almost abolished the agglutination titer, however, the decrease in opsonic activity was not as dramatic. A concentration of 0.03 mg/ml of the reduced and blocked γ M-antibody was required to give 50% phagocytosis in one hour.

c) Placental passage. Transport of immunoglobulins across the placental membrane is a highly selective process. The selection is not due to size differences but to structural differences among the immunoglobulins. Human γ G-globulin is freely transported across the membrane while neither γ A- nor γ M-globulin crosses this barrier,

(Franklin and Kunkel, 1958; Gitlin, et al., 1963). Brambell, et al. (1960) found that there was a differential in the rates at which papain fragments of rabbit γ G-globulins were transported across the placenta. The Fc fragment (newer nomenclature for Porter's Fragment III of rabbit γ -globulin, [Porter, 1959]) seems important for γ G-globulin transport since it crosses the placenta 11 times more rapidly than does the Fab fragment (newer nomenclature for Porter's Fragments I and II, [Porter, 1959]), but not quite as rapidly as intact γ G-globulin. Contrary to findings with humans, Hemmings and Jones (1962) found that immunization of pregnant rabbits with human red blood cells resulted in hemagglutinins of equal titer in both the maternal and fetal circulation. In both cases, the hemagglutinins were restricted to that region of a sucrose gradient after centrifugation in which one would expect to find macroglobulin but not 7S γ -globulin.

d) Skin and smooth muscle sensitization. The 'skin fixation' of an antibody depends on its ability to remain localized at an intradermal injection site in guinea pig skin and subsequently cause an anaphylactic reaction at the site when the antigen is administered systemically. This technique is commonly called passive cutaneous anaphylaxis (PCA). The ability of γ G-globulin of other species to become 'fixed' in the guinea pig skin, while γ A- and γ M-globulins of heterologous species do not possess this property, again seems to reside in the Fc fragment portion of the active molecule. The Fc fragment can block the fixation of intact γ G-globulin while Fab fragment cannot (Ovary and Karush, 1961). The inability of human γ M-antibody to fix in the guinea pig skin while γ G-antibody to the same

antigen did become fixed was shown by Ovary, et al. (1960). It has also been found that normal human γ A-globulin as well as γ A-myeloma proteins would not sensitize guinea pig tissues (Franklin and Ovary, 1963).

Genetic Control and Cellular Synthesis. Two sets of alleles controlling the light chains of human and rabbit γ G-globulins respectively have been useful as tools for study of genetic control of immunoglobulin synthesis. The class specific antigen sites are located on the heavy chain portion of the γ -globulin molecule. Since there are three such class-specific chains in humans, that is, γ , α , and μ chains, and two human light chain types, K and λ , there are six possible combinations of light and heavy chains for human immunoglobulin molecules. The six possible combinations have been identified in normal human serum. The various pairs are: γ K, $\gamma\lambda$, α K, $\alpha\lambda$, μ K, and $\mu\lambda$ (Fahey and Goodman, 1964). In recent studies, Pernis and Chiappino (1964); Pernis, et al. (1965); and Bernier and Cebra (1964, 1965), found that individual cells of human spleen contained only one class of heavy chain and one type of light chain.

In the rabbit spleen it was shown that a majority of the cells (63% to 78%) that stained with a fluorescent anti-light chain also were stained by an anti- γ chain that had been coupled with a contrasting fluorochrome (Cebra and Goldstein, 1965). A large part of the remaining fluorescing cells (14% to 21%) were stained by a fluorescent reagent specific for μ chains (Cebra, Colberg, and Dray, 1966). Also, it has been reported by Schoenberg, et al. (1965), that the synthesis of γ M-antibody in the rabbit was associated with a non-phagocytic mononuclear cell while the γ G-antibody to the same antigen

(diphtheria toxoid) was synthesized in typical plasma cells. Thus, γ M-globulin appears to be synthesized in cells separately from the other classes of immunoglobulins.

Sequential Appearance of Different Classes of Antibody During an Antigenic Stimulation. The sequence of the synthesis of the various classes of antibodies has been investigated using various systems. A thorough study by Bauer, et al. (1963) used the bacteriophage T2 and key-hole limpet hemocyanin to stimulate rabbits antigenically. The sequence of synthesis and secondary response was similar with both antigens, therefore a description of only one set of results is necessary. Antibodies were detected as early as six days after a single injection of the antigen, 90% of which were associated with γ M-globulin as defined by its elution characteristics from a DEAE-cellulose column. The proportion of antibody eluted in the γ G-globulin region continued to increase with time while that eluted in the γ M-globulin region decreased so that by day 16 the activity was about equally distributed between the two classes. Some 60 days after injection of the antigen more than 99% of the antibody activity was eluted in the γ G-globulin region. Restimulation of the rabbits at day 50 resulted in a marked increase in both classes of antibodies, however, the absolute amount of γ M-antibody neither exceeded that produced by the initial stimulation nor did it reach its maximum more rapidly. The level of γ G-antibody increased rapidly, reaching a maximum several fold greater than that previously reached and in a shorter interval. The levels of γ M-antibody reached following the secondary injection accounted for only a small fraction of the total activity of the serum.

Smith (1960) investigated the immune response of more than 150 premature and full term human infants. It was found that the infants' anti-Salmonella typhi agglutinin titers against the H antigen approximated those of the immunized mothers, however, in only four cases did the infants appear to passively receive anti-O antibodies from the mother. Subsequent immunization of infants having a H or O titer less than 1:5 with commercial 'triple typhoid vaccine' produced an increase of H agglutinin titers but no increase in O agglutinin titers even after repeated multiple injections. The H agglutinin was found to be sensitive to 2-mercapthoethanol and to have a sedimentation coefficient equal to or greater than 19S as determined in the Yphantis-Waugh partition cell (Waugh and Yphantis, 1953).

Evidence now available indicates that this sequential appearance may be more apparent than real. The methods of assay of the γ M- and γ G-antibodies are several fold more sensitive to detection of γ M-antibodies than γ G-antibodies. Using techniques to minimize the difference in detection limits, Altmeier, et al. (1965) have reportedly reduced the time difference between detection of γ M-antibody and detection of γ G-antibody to as little as two days.

On a cellular level, Schoenberg, et al. (1965) found that the appearance of cells of rabbit spleen making a particular class of specific antibody paralleled the appearance of the corresponding circulating antibody. In rabbits stimulated by a single injection of diphtheria toxoid there first appeared only cells producing γ M-antibodies. There was a slow increase of plasma cells making γ G-antibody which was evident around day 13 and which continued while the relative proportion of cells producing γ M-antibody decreased.

Phylogeny of the Immune Response. Investigations of the ability of invertebrates to respond to an antigenic stimulus with production of a protein analogous to mammalian antibody have yielded only negative findings. It would appear that phagocytosis together with intracellular digestion is the main line of resistance by invertebrates to infectious agents.

Thus, present evidence indicates that the antibody response is restricted to the vertebrates, and that some of the lowest forms seem to be capable of forming some type of antibody. Papermaster, et al. (1962, 1963) has presented evidence that would suggest that development of adaptive immunity was correlated with the development of thymus and lymphoid tissue. Serum proteins having the electrophoretic properties of mammalian γ -globulin have not been demonstrated in the lowest vertebrates, however, there are proteins that migrate in the β , or fast γ region (Papermaster, et al., 1962). The presence or absence of proteins migrating in the γ -globulin region cannot itself be used as *prima facie* evidence of the ability, or lack of ability, to produce antibodies. Elasmobranchs, frogs, and teleosts have been shown to produce antibodies that have a sedimentation coefficient of 19S and which are sensitive to mercaptoethanol, thereby resembling mammalian macroglobulin. After an extended period, following stimulation, cold blooded vertebrates begin to make antibodies that have a sedimentation coefficient of 7S (Uhr, et al., 1962; Gray, 1963). The 7S antibody differs from γ G-globulin of mammals in that it also was mercaptoethanol sensitive.

Marchalonis and Edelman (1965) found that the elasmobranch, Mustelus canis (smooth dogfish) could produce 17S, mercaptoethanol sensitive antibodies when immunized with hemocyanin. The serum also contained proteins with a 7S sedimentation coefficient that gave a reaction of identity with the 17S antibody in gel-diffusion plates. However, antibody activity in this 7S fraction could not be demonstrated.

Demonstration of an antibody response in the lower vertebrates is possible only if the correct antigen and reacting conditions are chosen. Thus, an absolute statement on the ability or inability of the invertebrates to produce antibodies may be unjustified at this time as the experimental conditions imposed upon the animal and/or the system used to detect its antibodies may not have been the correct ones for successful stimulation.

Role in Specific Immunity. Antibodies play an important role in a mammal's resistance to infectious agents. Pathogenic bacteria, following reaction with antibody, are dealt with in two ways which may or may not be interrelated. One of these, opsonization, has been discussed above. Briefly, a bacterium that has been opsonized with specific antisera is literally coated with antibody molecules, Interposition of the antibody molecules between the bacterium and the phagocyte facilitates engulfment of the bacteria by the phagocyte. At least part of the complement system may be required for maximum opsonization by a given amount of antibody (Boyden, et al., 1965). Briefly, the complement system is composed of some 10 to 11 serum proteins, some of which may act as enzymes (Becker, 1965).

The action of complement, in conjunction with antibody, is most dramatically demonstrated in its ability to kill, by lysis, certain bacteria. This bactericidal activity can be demonstrated in vitro and forms the basis of a very sensitive assay for the presence of small amounts of antibody to gram negative bacteria (Muschel and Treffers, 1956). The gram negative bacteria are especially susceptible to the bactericidal activity of specific antibody. Robbins, et al. (1965) used this assay system to compare γ G- and γ M-anti-S. typhimurium. It was found that the γ M-antibody more effectively sensitized S. typhimurium for lysis by complement. As little as 0.05 μ g γ M-antibody/ml caused detectable kill while 0.9 μ g γ G-antibody/ml (or 18 times more) was required to produce detectable kill.

Onoue, et al. (1965) compared the ability of rabbit γ G- and γ M-antibodies to the hapten p-azobenzenearsonate to sensitize sheep red blood cells for lysis with guinea pig complement to which this hapten had been coupled. These authors found that, on a weight basis, 30 to 50 times more γ G-antibody than γ M-antibody was required to achieve hemolysis of 50% of the cells in the test system.

Humphrey and Dourmashkin (1965) reported that sheep erythrocytes had a mean maximum number of binding sites for γ M-antibody of about 90,000 while there were about 600,000 sites to which γ G-antibody could bind. It was also reported that only two to three molecules of γ M-antibody were required to produce a hole in the erythrocyte membrane, while 2,000 to 3,000 γ G-antibody molecules were required. Calculations based on the above data and on the premise that one γ M-molecule, or two adjacent γ G-molecules will produce a hole, indicated that the

probability of having two γ G-molecules occupy adjacent sites would require numbers of molecules of γ G-antibody similar to those found experimentally, i.e. 2,000 to 3,000. The interpretation of the data of Onoue, et al., (1965) need not take into consideration different numbers of antigenic sites. Both the γ G- and γ M-anti-azobenzeneazobenzene are directed against the same antigenic determinant, therefore, on any conjugated cell there should be an equal number of sites available for both classes of immunoglobulins but there is still a substantial difference in the abilities of the two classes of molecules to sensitize for lysis.

Experimental evidence indicates that the complement-antibody mediated lesions that lead to cytolysis are common to all cell types (Green and Goldberg, 1960). The susceptibility of a wide variety of mammalian cells to destruction by complement in conjunction with specific antisera was summarized by Osler (1961). One would feel that the role of the various classes of immunoglobulins in mediating complement lysis of erythrocytes may also apply to the various nucleated mammalian cells.

The immunogen eliciting the antibodies used in this study was the polysaccharide capsule of Diplococcus pneumoniae, Type I. The polysaccharide, upon isolation from the pneumococci, was found to have a molecular weight of 171,000 (Record and Stacey, 1948), and to contain 60% uronic acid and 30% reducing sugars (Heidelberger, et al., 1936). The uronic acid was found to be D-galacturonic acid (Heidelberger, 1958).

MATERIALS AND METHODS

Immunization of the Horse. Hyperimmune serum was obtained by immunizing a horse with saline suspensions of heat-killed Diplococcus pneumoniae, Type I. The bacteria were initially passed through mice until they had become heavily encapsulated. Figure 1 shows the pneumococci with their rather large capsule stained with fluorescein labelled γ M-antibody. Cultures were then grown in brain heart infusion broth (BHI, Difco) enriched to contain 1% sheep blood. The bacteria were harvested, washed free of growth medium, suspended in 0.9% saline, and then heated to 60°C for one hour. The horse (500 kg) received a primary stimulation with three 30 mg portions of the heat-killed pneumococci given intramuscularly (rump) on days 1, 20, and 29. Sera from trial bleedings obtained were found to have a low agglutinating titer and showed only a trace of precipitins up to 48 days after the initial injection. An injection of 120 mg of bacteria was then made into the jugular vein on day 48. Thereafter, intravenous injections of 120 mg of bacteria were made on days 63, 97, 113, 118, and 158. A copious bleeding, 3 l, was taken 15 days after the first intravenous injection and the serum was found to contain 1.63 mg of precipitating antibody per ml of serum. Subsequent bleedings of 3 l each were taken on days 97, 113, 118, 158, 165, 180, 219, 362, and 431. The serum from each bleeding was titered for antibodies that precipitated with the Type I polysaccharide before and after reduction. Each 3 l bleeding yielded 1.2 l to 1.5 l of serum.

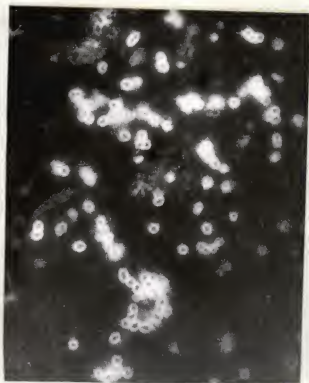


FIGURE 1: Diplococcus pneumoniae, Type I, with its polysaccharide capsule stained with fluoroscein conjugated γ M-antibody.

The precipitating antibody level has remained at about 1.5 mg/ml of serum over this period of 431 days after having reached a peak titer of 2.4 mg/ml at day 97. There has been only a slight increase in the level of precipitins that are resistant to reduction by 0.1 M mercaptoethanol, presumably γ G-antibody. At most, these resistant antibodies account for 18% of the total specific antibody precipitated by the polysaccharide. Figure 2 shows the precipitin curves for selected representative bleedings obtained using untreated and reduced serum.

Soluble Antigens

Preparation of Type Specific Polysaccharide. *D. pneumoniae*.
Type I were grown for 24 to 36 hours in 4 l batches of BHI enriched to contain 1% sheep blood. The bacteria were then harvested by centrifugation and washed several times with saline to remove traces of the medium. The bacteria were then suspended in 1 l of 0.15 M, phosphate buffered saline, pH 7.2, and deproteinized by several cycles of mixing with chloroform in a Waring blender. The clear aqueous layer containing the polysaccharide was concentrated ten to twenty-fold by pressure dialysis against distilled water. The polysaccharide was then precipitated by making the aqueous solution 1.3 M in sodium acetate and then by adding 1.5 volumes of 95% ethanol. After two further precipitations, the polysaccharide was lyophilized from a distilled water solution. The material appeared relatively homogenous by sedimentation velocity analysis and had a sedimentation coefficient of 3.0S. Hereafter, the polysaccharide from Type I pneumococci will be referred to as SI.

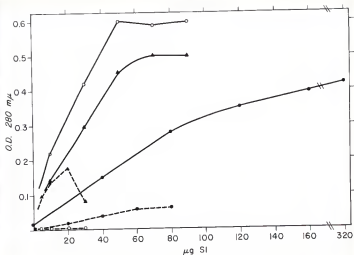


FIGURE 2: Quantitative precipitin curves obtained for sera taken at various times after initiation of immunization with Type I pneumococci. The sera used for the precipitin curves were collected from bleedings taken on days 97 (O), 165 (□), and 431 (△). Precipitin curves obtained following reduction of the sera with 0.1 M mercaptoethanol are indicated by the broken lines. The amount of SI indicated on the abscissa was added to 0.5 ml of untreated or reduced antiserum. The washed precipitates were dissolved in 1.0 ml of 0.1 N NaOH for optical density measurements at 280 mμ.

Trace labelling SI with Radioiodine ^{125}I (^{125}I - SI). The SI was extrinsically labelled with ^{125}I by the following procedure: 100 mg of SI was mixed with 500 mg triphenylphosphorus (Peninsular Chem. Research, Gainesville, Fla.), 9 mg KI and 2 mg ^{125}I (New England Nuclear, Boston, Mass.). All moisture was removed by gentle heating. Anhydrous benzene (40 ml) was added and the reaction mixture was heated and allowed to reflux for 72 hours. The benzene phase was decanted and the solids were dissolved in 5 ml water and washed into a suitable container. The polysaccharide was then precipitated by the addition of an equal volume of 95% ethanol. After standing in the cold for several hours, the precipitated polysaccharide was collected by centrifugation. After dissolution in 5 ml water, the polysaccharide was again precipitated with an equal volume of 95% ethanol as before. This dissolution-precipitation was continued until the supernatant fluid was found to be free of radioactivity. Following the removal of non-bound ^{125}I , the conjugate was dissolved in a small quantity of water, 5 ml to 10 ml, and dried by lyophilization. This same procedure was followed in the preparation of ^{125}I - labelled dextran, Type II (SII) pneumococcal polysaccharide, and human A-substance. The characteristics of these products are given in Results.

Isolation of Purified Antibodies

Horse Anti-SI Antibodies. A quantitative precipitin curve was obtained for the serum from each bleeding and these data were used to determine the amount of polysaccharide needed for precipitation of the maximum amount of antibody. The optimal amount of

SI was then added to a large volume of the serum and the mixture was incubated at 37°C for one hour and then at 2°C overnight. The precipitate was collected by centrifugation and washed twice with cold saline. The precipitate was then suspended in a quantity of 0.05 M acetate buffer, pH = 3.5, equal to one-half the original serum volume and allowed to stand at room temperature for one hour. The portion of the precipitate which did not dissolve was then removed by centrifugation. The supernatant now contained about 1 mg of protein from each ml of serum used. The extinction coefficient ($E_{280}^{1\%}$) of 15.0, found for horse γ M-globulin (McDuffie and Kabat, 1956), was used to determine protein concentrations. The protein eluted from the antigen-antibody precipitate was about 82% γ M-globulin while the balance consisted of immunoglobulins which sedimented with an $s_{20,w} = 6.4S$. This slower sedimenting fraction will be referred to as '6.4S immunoglobulins'.

Under the conditions used for eluting the antibodies from the SI, the SI remained insoluble. The insolubility of the SI was most probably due to the low pH of the eluting buffer and the antibody which was not eluted under these conditions. Direct evidence indicating an absence of solubilization of the SI include: Absence of immune precipitation upon restoration of physiological conditions and no evidence of soluble complexes, of antigen and antibody, upon ultracentrifugal analysis. If soluble SI was present in the antibody solution it would be removed by retardation on the Sephadex-G-200 column.

Separation of γ M-antibody from the '6.4S Immunoglobulins'.

The technique of molecular sieving in columns of Sephadex G-200 (Pharmacia), equilibrated in 0.1 M phosphate buffer, pH 6.8, was used to separate the various immunoglobulins. The proteins that had been eluted from the polysaccharide were equilibrated with the same phosphate buffer by dialysis and 4 ml portions containing about 10 mg of protein were applied to a column of Sephadex G-200 (2.5 cm x 60 cm). The proteins were eluted by passage of the phosphate buffer through the column. The effluent was monitored by measuring absorption at 280 m μ . Fractions of approximately 4.7 ml were collected.

Fractionation of '6.4S Immunoglobulins'. The pool of '6.4S immunoglobulins' from the above G-200 column were equilibrated with 0.01 M phosphate buffer, pH 7.5, and applied to a DEAE-cellulose column, 0.7 cm, i.d. x 14 cm, equilibrated in the same buffer. A homogeneous fraction of γ G-antibody was not retained by the column and was collected after one column volume of buffer had been passed. Further protein fractions were obtained by stepwise elution with this buffer which was made 0.05, 0.1, 0.2, and 0.3 M in NaCl. The fraction eluting at 0.3 M NaCl was found to be nearly homogeneous γ A-globulin or the 'T-globulin' of van der Scheer (1940).

Preparation of Normal Horse γ G-globulin. Serum was collected from a non-immunized horse and the globulins were precipitated by making the serum 37% saturated with ammonium sulfate. After standing in the cold overnight, the precipitated proteins were collected by centrifugation and washed several times with cold 37% saturated

ammonium sulfate. The precipitate was then dissolved in 0.00525 M phosphate-borate buffer, pH 8.4 (Press, et al., 1960), then dialyzed overnight against the same buffer. The soluble globulins were applied to a triethylaminoethyl-cellulose (Biorad Lab., Richmond, Calif.) column equilibrated in the same buffer. That protein fraction which was not retained by the column was concentrated by pressure dialysis and contained only γ G-globulin.

Reduction of Immunoglobulins

Depolymerization of γ M-globulin to Subunits Using Monothiois.

The proteins of whole serum which were susceptible to reduction were reduced by making the serum 0.085 M in mercaptoethanol (Morton and Deutsch, 1957) followed by incubation at 37°C for 30 minutes or, alternatively, by making the serum 0.1 M in thioglycolate (Sigma Chemical Co., St. Louis, Mo.) followed by incubation for one hour at 37°C. The free sulphydryl groups were blocked by alkylation with a 10% excess of monoiodoacetamide (Mann Research Labs, New York, N. Y.) which was added following reduction. The excess reagents were removed by dialysis against 0.1 M phosphate buffer, pH 6.8. Reduction of γ M-antibody was carried out by adding the desired amount of a stock solution of 0.85 M mercaptoethanol or 1 M thioglycolate to the protein in a 0.1 M phosphate buffer, pH 6.8, and proceeding as for whole serum. Concentration of the stock solutions of reducing agent were determined by titration with 5,5-dithiobis-(2-nitrobenzoic acid) as described below.

Depolymerization of γ M-globulin Using Dithiothreitol (DTT).

The principal advantage of using DTT is that much lower concentrations

of DTT are needed to depolymerize the γ M-globulin. The greater effectiveness of DTT is a consequence of the structure of the molecule as described by Cleland (1964). Reduction of a disulfide bond by the previously described thiols required the participation of two molecules of the thiol. The first step in the reduction involves the formation of a mixed disulfide (between the protein and the thiol) and one free -SH group on the protein. This step is followed by a reaction of the mixed disulfide with a second thiol molecule to yield a second free sulfhydryl group on the protein. DTT circumvents necessity for the participation of a second thiol molecule since each DTT molecule contains two sulfhydryl groups and the proper configuration to complete the reduction (Figure 3). Depolymerization of γ M-globulin with DTT was accomplished by adding the appropriate volume of a solution of DTT that was approximately tentimes more concentrated than that finally required. Again the concentration of this solution was determined by titration. The reduction mixture was then incubated at 37°C for one hour.

Re-aggregation of γ M-subunits by Reoxidation.

a) The γ M-antibody (2.5 mg/ml) was reduced in 0.1 M phosphate buffer, pH 6.8, at a final concentration of 0.1 M thioglycolate (Sigma Chemical Co., St. Louis, Mo.) upon incubation for one hour at 37°C. Complete reduction to subunits was confirmed by alkylation of a small portion of the reaction mixture and subsequent sedimentation analysis. The balance of the reaction mixture was passed through a column of Dowex 1 x 8 acetate equilibrated in 0.1 M sodium acetate. The subunits were then allowed to reassociate while being concentrated

to 5 to 7 mg/ml by pressure dialysis against 0.14 M phosphate-borate buffer, pH 8.0.

b) The γ M-globulin (6 mg/ml) was reduced with 0.0025 M DTT (0.005 M in -SH) at 37°C for one hour. Complete reduction to subunits was confirmed by sedimentation analysis. Reassociation was then allowed to proceed while the subunits were dialyzed against 0.00525 M phosphate-borate buffer, pH 8.4, containing 0.002 M Versene.

Separation of Polypeptide Chains. Either γ G- or γ M-globulin was equilibrated with 0.55 M Tris (Tris [hydroxymethyl] aminomethane) buffer, pH 8.2. The solution was then made 0.75 M in mercaptoethanol to reduce the globulin which was then alkylated, and fractionated on a column of Sephadex G-100, equilibrated in 1 M propionic acid, according to the method reported by Fleishman, et al., (1962).

Specific Precipitation and Coprecipitation of SI. Data for quantitative precipitin curves were obtained by adding increasing volumes of a 1 mg/ml solution of SI to 0.5 ml of the test serum or a saline solution containing 0.5 mg of purified γ M-antibody. The mixtures were incubated at 37°C for one hour and then at 2°C overnight. The precipitates were collected by centrifugation, washed twice with cold saline, and then dissolved in a convenient volume of 0.1 N sodium hydroxide. The protein present in the precipitate was determined by optical density measurements at 280 m μ .

The amount of ^{125}I - SI required to give the maximum amount of precipitate with a given amount of intact γ M-globulin and the amounts of rabbit antiserum (anti-horse immunoglobulins) required to precipitate maximally a given amount of γ M-antibody or normal γ G-globulin

(and '6.4S immunoglobulins') were determined separately in advance. For quantitation of the amount of labelled polysaccharide that was coprecipitated, 0.5 mg amounts of intact γ M-antibody, reduced and alkylated γ M-antibody, normal γ G-globulin, '6.4S immunoglobulin' or other horse globulin preparation were mixed with the amount of ^{125}I - SI that was optimal with respect to the same weight of intact γ M-antibody. Each globulin solution was set up in triplicate with SI. After incubation of the reaction mixtures for one hour at 37°C precipitation could be seen only in the tubes containing intact γ M-antibody or '6.4S immunoglobulins'. At this time, that amount of rabbit anti-globulin required to precipitate maximally the horse immunoglobulin in a given tube was added to each tube with the exception of one set of tubes which served as a control for amount of polysaccharide which could be directly precipitated by intact γ M-antibody. After further incubation at 37°C for one hour and at 4°C overnight, the precipitates derived from each immunoglobulin were dissolved in 1.0 ml Hyamine and placed in vials with 5.0 ml of scintillation fluid (Kinard, 1957) and counted for ten minutes in a liquid scintillation counter (Packard Instrument Co., Series 314A). The third precipitate from each set was dissolved in 0.1 N sodium hydroxide and the optical density at 280 m μ was determined.

Immunodiffusion, Immuno-electrophoresis, and Radioimmuno-electrophoresis. The various immunodiffusions and immuno-electrophoreses were carried out on glass slides (either 1" x 3" or 2" x 3") covered with a 2 mm layer of 0.8% Ionagar made up in 0.05 M Veronal buffer, pH 8.6. Electrophoresis was for 2.5 hours at 3 ma/cm (width).

For radioimmuno-electrophoresis, slides prepared as above and the same conditions of electrophoresis were used. These slides were developed by filling the troughs with a 1 mg/ml solution of ^{125}I - SI plus the appropriate goat or rabbit anti-horse globulin. The developed gel was washed in saline, then in water, and then dried and stained with amido black dissolved in a solution of four parts water, four parts methanol, and one part glacial acetic acid. Radioautography was carried out by placing the slide in contact with Kodak Industrial X-ray film (Type KK) for one to three weeks. The technique of radioimmuno-electrophoresis has been previously described by Hochwald, *et al.* (1961).

Preparation of Specific Reagent Sera

Goat Anti-horse γM -globulin. A goat was injected twice with two 9.25 mg amounts of γM -antibody given four weeks apart. For the first injection the antigen was incorporated in complete Freund's adjuvant and for the second injection, it was precipitated with alum. One-half of each antigen dose was injected intramuscularly into each hind leg. The goat responded with precipitating antibodies, the bulk of which reacted with the class specific portion of the γM -globulin molecule. A small amount of the antibody cross-reacted with the other immunoglobulins.

Rabbit Anti-horse Immunoglobulins. An anti-immunoglobulin serum with broad specificity was prepared by injection of the unfract ionated immunoglobulins that were eluted from the SI polysaccharide. About 3 mg of the globulins were emulsified in complete Freund's adjuvant and injected into multiple subcutaneous sites (Bernier and Cebra,

1965). Serum was collected two or three weeks after immunization. This antisera reacted with common antigenic sites as well as with the class specific sites of horse γ M-, γ G-, and γ A-globulins.

Rabbit Anti-Horse Light Chain. Light chain from either γ G- or γ M-globulin was emulsified with complete Freund's adjuvant and was injected at multiple subcutaneous sites as described above. The resulting antisera were specific for light chains.

Titration of Free Sulfhydryl Groups. The colorimetric method for the determination of sulfhydryl groups has been described by Ellman (1959). An appropriate dilution in 0.1 M Tris acetate, pH 8.0, 0.002 M EDTA of the material to be titrated was mixed with 0.02 ml of 0.01 M 5,5-dithiobis-(2-nitrobenzoic acid), (DTNB, Aldrich Chemical Co., Milwaukee, Wisc.). To titrate sulfhydryl groups on proteins the solution was made 0.5% in sodium dodecylsulfate. The final volume was adjusted to 3.02 ml and the reaction mixture allowed to stand at room temperature for 30 minutes. The optical density was then determined at 412 m μ . The concentration of sulfhydryl groups could be calculated from the molar extinction coefficient of 13,600 for the thiophenol, the reduced form of the reagent DTNB. The number of disulfides reduced in γ M-antibody were determined by reducing the molecule with the desired concentration of dithiothreitol. The reaction was terminated by lowering the pH to 2.5 with 2 N HCl. Excess reducing agent, as well as by-products of the reduction, were removed by passing the reaction mixture through a column of Sephadex G-25 equilibrated with 0.15 M NaCl brought to pH 2.5 with HCl. The fraction containing the highest protein concentration, as determined by

optical density at 280 m μ , was then used to titrate the free sulfhydryls. The extent of polymerization was determined by chromatography on Sephadex G-200.

Preparation of Fluorescent γ M-antibody. Two ml of buffer containing 11.4 mg of γ M-antibody were equilibrated with 0.01 M phosphate, 0.05 M NaCl, pH 7.5 by dialysis and the solution was then placed in an ice bath. The pH was adjusted to 9.3 with sodium hydroxide and maintained at this pH with a Radiometer Autotitrator (Radiometer Corp., Copenhagen, Denmark) during the first two hours of conjugation. A 140 μ g amount of fluorescein isothiocyanate (Baltimore Biological Labs., Baltimore, Md.) was dissolved in a very small volume of acetone and was added to the protein solution. The mixture was stirred constantly for the initial two hours of reaction and placed at 2°C overnight. The reaction mixture was then diluted 1:10 with the pH 7.5 buffer and applied to a DEAE-cellulose column (2.5 x 40 cm) equilibrated with the 0.01 M phosphate buffer 0.05 M in NaCl. Components were eluted using stepwise increases in the NaCl content of this buffer. The fraction eluted with 0.2 M NaCl in the 0.01 M phosphate buffer, pH 7.5, had optical densities at 280 m μ and 495 m μ in the ratio of 3.2:1 and was found to give very good staining with little or no non-specific staining. The chromatographic fractionation of the conjugate was adapted from Cebra and Goldstein (1965).

Antibody Assay Using Agglutination of SI Coated Erythrocytes. Washed sheep erythrocytes were sensitized for agglutination using the method suggested by Askonas, et al., (1960). Sheep red blood

cells (0.2 ml) that had been previously washed three times with saline buffered at pH 7.4, were suspended in 10 ml of buffered saline and then 0.2 ml of a filtrate from an 18 to 24 hour culture of Type I pneumococci was added. After the mixture had incubated at 37°C for 45 minutes, the cells were washed three times with buffered saline containing 1% Bovine Serum Albumin (Armour, Chicago, Ill.) and then were finally suspended in 20 ml of buffered saline.

Hemagglutination was then carried out by preparing two-fold serial dilutions of the antibody preparation or antiserum in plastic trays having multiple depressions. To 0.2 ml of each dilution was added 0.1 ml of the cell suspension. The mixture was allowed to stand at room temperature for two hours and the agglutination patterns were recorded. It was found that readings taken at two hours were not appreciably different from those taken at the end of 12 to 18 hours.

Mouse Protection. Young adult mice obtained from the Charles River Farm, Massachusetts, were used for in vivo assay of various antibody preparations. In each case, six hour cultures of highly virulent D. pneumoniae, Type I, were diluted with 0.9% saline to a concentration of 1,000 organisms/ml as determined in a particle counter (Coulter Electronics, Hollywood, Fla.). Equal volumes of this suspension and the antibody preparation to be tested were mixed and 0.2 ml portions were injected into the mice intraperitoneally; thus, each mouse received 100 organisms. The animals were observed for a 36 hour period and the number of deaths were recorded.

RESULTS

Immunoglobulin Response of the Horse. The initial antibody response of the horse immunized with Type I pneumococci was described in a Master's Thesis by the author (Hill, 1964). At the time the thesis was completed, only data of bleedings taken through day 180 were available (Figure 2). Since then, the horse has been bled numerous times with no additional antigenic stimulation since day 158. The level of circulating antibodies, as determined by quantitative precipitin tests, has decreased slightly from 2.4 mg/ml determined for the bleeding of day 97, to 1.5 mg/ml, where it has remained for 31 months. Continued evaluation of the proportion of antibody in the serum that retained its ability to precipitate SI following treatment with mercaptoethanol has not indicated more than a slight increase of these resistant antibodies (γ G- and γ A-immunoglobulins). Thus, it appears that this horse will probably not show the conversion to predominately γ G-antibody production that is characteristic of many mammals' response to various immunogens.

Elution of the antibodies from an antigen-antibody precipitate at a low pH, as described in Materials and Methods, led to the isolation of a mixture of antibodies of different classes. The majority of the antibodies were of the γ M-globulin class as evidenced by sedimentation analysis (Figure 7A,) and immunoelectrophoretic analysis (Figure 5A). Of the total quantity of antibodies eluted from the

precipitate, 82% was determined to be γ M-globulin by gel filtration on Sephadex G-200 (Figure 4). In Figure 5B one can see that the mixture of 6.4S components were capable of precipitating SI. A detailed examination of the components that comprised the smaller immunoglobulin eluted along with the γ M-antibody and resolved from it on Sephadex G-200, indicated that this fraction was composed of two populations of globulins (Figure 6A), differing in average net charge as reflected by their different electrophoretic mobilities (Figure 5A). Their difference in charge was subsequently utilized to separate the components of the '6.4S immunoglobulins' by ion exchange chromatography on a column of DEAE-cellulose. The molecules that were not retained on the column under the starting conditions, i.e. pH 7.5, 0.01 M phosphate buffer, were found to have the electrophoretic properties ascribed to γ G-globulin when examined by immunoelectrophoresis (Figure 5C). A later fraction, eluted from the column when the sodium chloride concentration of the buffer was increased to 0.3 M had an electrophoretic mobility somewhat faster than the γ G-globulin (Figure 5C). In fact, its mobility approximated that of the previously described horse 'T'-globulin, and, in accord with the newer nomenclature, it will be referred to as γ A-globulin (Rockey, et al., 1964).

The Antigenic Composition of Horse Anti-SI Immunoglobulins.

An antigenic analysis of the various immunoglobulin classes described above showed that certain antigenic determinants were shared among all three classes. These common determinants are expressed as reactions of partial identity in double gel diffusion

plates as shown in Figures 6A and B. In Figure 6A one can see the relatively sharp, single precipitin band formed as the γ M-antibody (well labelled γ M) reacted with its antiserum (labelled A-Ig). The same antiserum (A-Ig) reacted with components in the well containing the fraction from the G-200 column that was eluted after the γ M-antibody, labelled 6.4S. The band closest to the A-Ig well was formed by γ G-globulin as evidenced by its complete fusion with the band formed between A-Ig and chromatographically pure γ G-globulin (γ G). Where this band meets the upper γ M-antibody band there is partial fusion, or a reaction of partial identity. However, there is also a spur that appears on the opposite side of the γ M-antibody band, indicating the presence on the γ G-antibody of antigenic sites that do not occur on the γ M-antibody. The precipitin band nearest the '6.4S' well was formed by the γ A-globulin mentioned in the previous section. Here the band was formed by precipitation due to antibodies directed against the class-specific sites on the molecule, the antibodies reacting with the common sites having been effectively removed by precipitation with γ G-antibody. Hence, the cross reaction with the γ M-antibody band is less apparent, but the reaction of its unique sites is more apparent. One also can notice that the γ M-antibody band spurs through both the γ G- and γ A-antibody bands, indicating the existence of sites peculiar to γ M-immunoglobulin alone.

Figure 6B is an antigenic analysis of the fractions obtained from the '6.4S immunoglobulins' using DEAE-cellulose chromatography. It can be seen that the protein that was not retained on the column

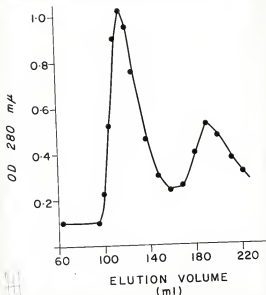


FIGURE 4: Resolution of γ M-antibody and '6.4S immunoglobulins' on Sephadex G-200. The protein which was eluted after the passage of one column volume of buffer was solely γ M-antibody while that eluted later constituted the '6.4S immunoglobulins'. The immunoglobulin mixture was applied to a 2.5 cm x 100 cm reverse-flow Sephadex Laboratory Column, filled with Sephadex G-200 equilibrated in 0.1 M phosphate buffer, pH 6.8.

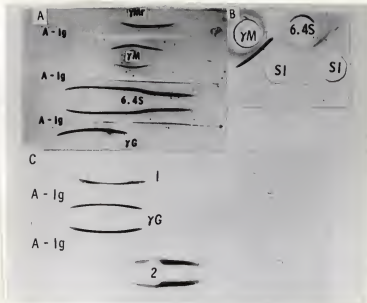


FIGURE 5: Immuno-electrophoretic analysis of the horse anti-SI components and their reaction with SI. Plate A shows that the γ M-antibody (γ M) chromatographed on Sephadex G-200 was free of other immunoglobulins recognized by the anti-immunoglobulin serum (A-Ig). However, the '6.4S' component (6.4S) contained two electrophoretically distinct populations of molecules, one of which migrated as γ G-globulin (γ G). Plate B shows that the isolated γ M-antibody and the 6.4S components were capable of forming precipitin bands with SI. Plate C shows the separation of the two electrophoretic populations of the '6.4S' component on DEAE-cellulose. The fraction labelled 1 was eluted with 0.01 M phosphate buffer, pH 7.5, while a later fraction was eluted with the same buffer which had been made 0.3 M in NaCl, (labelled 2).

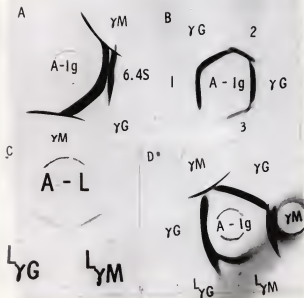


FIGURE 6: The antigenic analysis of horse anti-SI immunoglobulins and their isolated 'light' polypeptide chains. See text for explanation.

under the starting conditions (here labelled 1) shows a reaction of identity with the γ G-globulin (γ G). The protein that was eluted with the buffer made 0.2 M in NaCl (here labelled 2) shows a reaction of partial identity with the γ G-globulin. The proteins in '2' form a spur over the γ G-band, however, while the γ G-band does not form a spur through '2'. This indicates that '2' contained both γ G- and γ A-immunoglobulins. The protein eluted at 0.3 M NaCl (3), forms a precipitin line which passes through the line formed by γ G-globulin (double spurring). Thus, the protein in '3' has protein molecules which possess antigenic determinants that are different from γ G-globulin and conversely, γ G-globulin has unique sites. These two proteins also share sites and these are evidenced by the reaction of partial identity that also occurs between these two bands. Thus, the second component that 'made-up the '6.4S immunoglobulins' along with γ G-antibody, was antigenically distinct from γ G-antibody and was γ A-antibody, based on its electrophoretic properties.

The reaction of partial identity that has been described as occurring between the different classes of immunoglobulins can be in part interpreted as indicating that certain polypeptide chains are present in all three immunoglobulins. Small polypeptide chains, called light chains, were isolated from γ G-globulin and γ M-antibody using the system devised for isolation of light chains from rabbit γ G-globulin. In Figure 6C one can see that light chains from γ G-globulin ($L_{\gamma G}$) and from γ M-antibody ($L_{\gamma M}$) form precipitin bands which show complete reactions of identity when diffused against an anti-light chain serum (A-L). The same reaction was present if the

A-L was anti-L_{γM}, anti-L_{γG} or a mixture of the two. Thus, as far as this system can detect, the light chains from the two immunoglobulins are identical. The isolated light chains show a reaction of partial identity with either γG-globulin or γM-antibody and in each case the intact immunoglobulin forms a band which shows a spur beyond the light chain band (Figure 6D).

Physicochemical Properties of γM-antibody. Following Treatment with Reducing Agents

Reduction with Monothiols. The immunoglobulins of the γM-class are uniquely susceptible to depolymerization by reduction under rather mild conditions. The conditions generally employed call for incubation times varying from 30 minutes to 48 hours in the presence of 0.1 M 2-mercaptoethanol, thioglycolate, mercaptoethanolamine or one of several other thiols.

For a better understanding of the process of depolymerization of the γM-antibody, it seemed desirable to carry out the reduction under conditions as mild as possible, that is, using minimal concentrations of reducing agent for short time intervals (15 to 60 minutes) and at a pH around neutrality. To determine accurately the lowest concentration of reducing agent that could be used to effect depolymerization, a stock solution of mercaptoethanol (standardized by titration) was used to make a series of solutions of γM-antibody 0.04 M, 0.06 M, 0.075 M, and 0.1 M in reducing agent. The solutions were incubated at 37°C for 30 minutes, the free sulfhydryl groups were then blocked with iodoacetamide to stop reduction, and the extent of depolymerization was estimated by

sedimentation analysis (Figures 7B and C). Under these conditions, depolymerization of all of the molecules of γ M-antibody was not achieved in solutions having a concentration of mercaptoethanol below 0.075 M.

Subsequently, concentrations of 0.085 M mercaptoethanol, and incubation for 30 minutes at 37°C has been used to give maximum yield of subunits. γ M-antibody depolymerized under the above conditions, or any other set of conditions tried, that resulted in the formation of 6.1S subunits, was no longer capable of precipitating SI (Figure 8A). Attempts to demonstrate that depolymerization of the γ M-antibody by reduction had exposed or produced new antigenic sites or separated groups of sites present in the intact molecule were not successful. The subunits completely cross-reacted with intact γ M-antibody when tested in gel diffusion with either goat or rabbit anti-horse γ M-globulin (Figure 8B). Thus, it appeared that the subunits, which no longer could precipitate antigen, each still contained a set of antigenic sites representative of those which could be detected on the whole molecule. Concentrations of mercaptoethanol around 0.1 M exceed the level of reducing agent found sufficient to free a significant portion of the light chains from covalent bonding with heavy chains in horse γ G-globulin (Fleishman, et al., 1963). Thus, there seemed to be a possibility that the subunits produced from γ M-antibody contained light chains which had been freed from covalent bonding with μ -chains during the process of depolymerization.

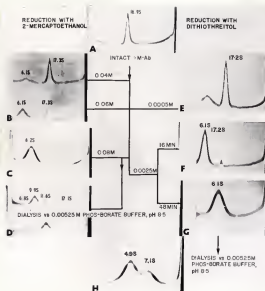


FIGURE 7: Depolymerization of γ M-antibody by reduction with two different thiol compounds used at varying concentrations and reaggregation of the subunits by reoxidation. A. γ M-antibody purified on a column of Sephadex G-200; B. γ M-antibody reduced with 0.04 M (upper) and 0.06 M (lower) mercaptoethanol; C. γ M-antibody totally depolymerized by 0.08 M thioglycolate; D. Reaggregation of 'C' by reoxidation; E. γ M-antibody only partially depolymerized by reducing 10.7 disulfide bonds with 0.0005 M dithiothreitol; F and G. γ M-antibody almost completely depolymerized with 0.0025 M dithiothreitol which cleaved 22 disulfide bonds per molecule, F at 16 minutes and G at 48 minutes after reaching 56,100 rpm; H. Aggregates formed by reoxidation of above.

Reduction with Dithiothreitol (DTT). Reduction of protein disulfides with DTT is a reaction that proceeds much more rapidly and requires much lower concentrations of reducing agent than reduction of the same number of disulfide bonds with the monothiols described above. γ M-antibody was reduced to its subunits after incubating it for one hour at 37°C in the presence of 0.005 M DTT (0.01 M sulfhydryl group), as detected by ultracentrifugal analysis. Again, the subunits prepared under these mild conditions could not directly precipitate antigen.

Reduction of a 5 mg/ml γ M-antibody solution with only 0.0025 M DTT under the conditions used above, resulted in about 70% of the γ M-antibody being depolymerized to subunits. The scheme of a relatively complete study of the reduction of the molecule under these conditions is outlined in Figure 9.

One portion (7.5 mg) of γ M-antibody reduced with 0.0025 M DTT, was brought to pH 2.5 immediately after incubation to prevent further reduction and was then passed through a column of Sephadex G-25 to remove the reducing agent from the protein. The peak protein fraction, as determined by optical density measurement at 280 m μ , was used to titrate the free protein bound sulfhydryl groups. The fraction was found to be 6.6×10^{-7} M in γ M-antibody (using 940,000 as the m.w. of γ M-globulin) and to be 2.89×10^{-5} M in free sulfhydryl groups. On a molecular basis there were 44 free sulfhydryl groups per γ M-molecule or 22 disulfide bonds had been broken per molecule by the DTT. This was in direct agreement with a similar study of human γ M-globulin, which could be reduced to

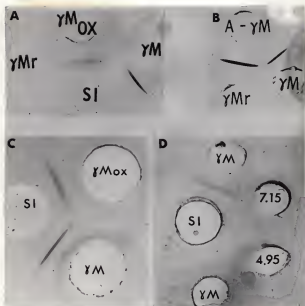


FIGURE 8: Antigenic relationship and antibody activity of γM -antibody, its subunit and products of reoxidation. In plate A it can be seen that γM -antibody (γM) and the mixture of products of reoxidation (γM_{ox}) form precipitin bands with SI, however, the reduced, alkylated subunits (γMr) do not precipitate SI. Plate B shows that the subunit (γMr) remains antigenically identical to intact γM -antibody. Plate C shows another reoxidation that resulted in components capable of precipitating SI. Plate D shows that neither the 7.3S nor the 4.9S subunits of γM -antibody could precipitate SI in gel.

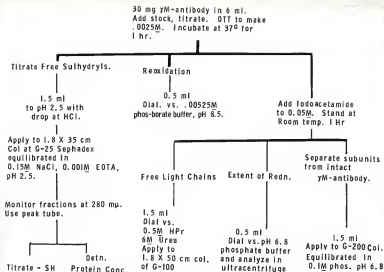


FIGURE 9: A scheme for the study of the effect of 0.0025 M Dithiothreitol on γ M-antibody. Parameters covered in each step are indicated in block letters.

subunits upon cleavage of 22 disulfide bonds with 0.01 M borohydride (Jacot-Guillarmod and Isliker, 1962).

Another portion of γ M-globulin (17.5 mg) which had been reduced in 0.0025 M DTT, was blocked by making it 0.05 M in 0.1 M phosphate buffer, pH 6.8. Ultracentrifugal analysis was used to obtain a rough approximation of the extent to which the γ M-antibody had been reduced to subunits (70%). A better estimation was obtained by gel filtration through a column of Sephadex G-200. From the elution diagram of the column, as monitored by optical density measurements at 280 m μ , it was determined that 57% of the γ M-antibody had been completely depolymerized to subunits.

The remaining 7.5 mg of the reduced and blocked antibody was equilibrated with a solution of 6 M Urea, 0.05 M in propionic acid and chromatographed on Sephadex G-100 to obtain an estimate of the amount of light chains that had been released from the molecule by the DTT. A clean separation of light chains was not obtained on the column as there was an unresolved component that chromatographed between the subunit and the light chains which was probably free heavy (μ) chain (Figure 10).

A five fold reduction of the concentration of DTT, that is to 0.0005 M, led to depolymerization of only a very small part of the total γ M-globulin present (Figure 7E); however, titration of the disulfide bonds broken indicated that an average of 10.7 disulfides had been broken per γ M-molecule. Thus, it appears that reductive depolymerization of macroglobulin requires cleavage of very resistant or very many disulfide bonds.

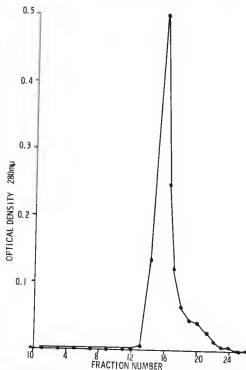


FIGURE 10: Release of free light chains by DTT. γ M-antibody was reduced with 0.0025 M DTT, alkylated with iodoacetamide, dialyzed vs 0.5 M propionic acid made 6 M in Urea. Gel diffusion of the various fractions against an anti-light chain serum showed precipitin reactions only in the trailing shoulder.

Reassociation of γ M-subunits by Reoxidation. Preliminary experiments to demonstrate reassociation of the reductively produced subunits of γ M-antibody were carried out by reducing the antibody with 0.1 M thioglycolate. The reducing agent was subsequently removed by passage of the reaction mixture through Dowex-1 acetate resin, which retained the thioglycolate. Reaggregation was accomplished by dialyzing the protein against 0.14 M phosphate-borate buffer, pH 8.0, while concentrating it two to three fold by pressure dialysis. Sedimentation analysis, following the spontaneous reoxidation, indicated boundaries having $s_{20,w} = 6.8S, 9.9S, 11.6S,$ and $17.1S$ (Figure 7D). This mixture of subunits in various stages of reaggregation was shown to be capable of precipitating with SI in agar gel whereas a portion of the γ M-antibody which had been acetylated following reduction did not precipitate with SI (Figure 8A).

At that ratio of subunit protein:SI giving maximum precipitation, as determined by quantitative precipitin curves, 42% of the reoxidized subunits could be precipitated or coprecipitated relative to the same weight of intact, untreated γ M-antibody. Thus, in this heterogeneous mixture, one was able to demonstrate the presence of a significant part of the precipitin activity in the reoxidized aggregates when compared to that activity present in the same weight of intact γ M-antibody. Solutions of γ M-antibody at concentrations of 5 to 6 mg/ml were reduced with 0.0025 M DTT as described in Materials and Methods. The extent of reductive fragmentation as estimated by sedimentation analysis, indicated that around 70% of

the protein was converted to subunits, the balance being intact molecules. Sedimentation analysis after three to five hours dialysis against the reoxidation buffer, 0.00525 M phosphate-borate buffer, pH 8.4, indicated that virtually all of the γ M-antibody was subsequently in the form of subunits. This further reduction was probably due to the continued presence of DTT and its increased activity (about ten fold) in the range of pH 8 (Cleland, 1964). Reoxidation was allowed to proceed for some 70 hours in the cold as the DTT was dialyzed away, following which the remaining free sulphydryls were blocked with iodoacetamide. A series of 12 to 15 reduction-reoxidation experiments were performed using DTT, and Figure 7H shows the sedimentation analysis of the products formed in one instance. Figure 11 shows representative sedimentation analyses which indicate the range of subunit aggregates formed by reoxidation. In instances when higher molecular weight aggregates were not obtained by reoxidation of subunits, the 'subunits' themselves showed a tendency to separate. It was found that the 6.1S subunit could then be resolved into two components following reoxidation. One of these sedimented with an $s_{20,w} = 4.9S$ while the second component had an $s_{20,w} = 7.2S$ (Figure 7H).

These two components were separated by chromatography on Sephadex G-200 and the elution pattern is shown in Figure 12. Neither of these components, nor a mixture of them, were capable of either precipitating SI, or of agglutinating Type I pneumococci;

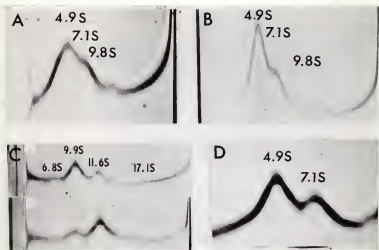


FIGURE 11: A series of ultracentrifuge frames from various reoxidation experiments showing the variable results obtained.

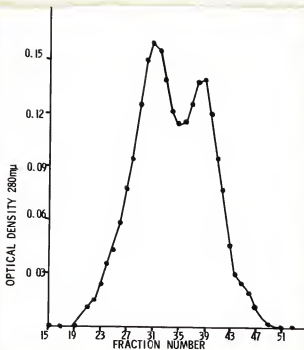


FIGURE 12: Separation by gel filtration on Sephadex G-200 of the 7.2S (leading part) and 4.9S (trailing peak) subunits of γ M-antibody. The respective molecular weights (as determined by the s and D method) are 180,000 and 100,000 suggesting the 4.9S subunit is one-half of the 7.2S subunit.

however, both rendered the pneumococci agglutinable by a goat anti-horse γ M-globulin serum indicating that at least a portion of the subunits retained antigen binding activity.

Molecular weight estimations based on sedimentation-diffusion measurements, as described by Svedberg and Pedersen (1940), indicated approximate molecular weights of 100,000 for the smaller component ($s_{20,w} = 4.9S$; $D_{20,w} = 3.82 \times 10^{-7} \text{ cm}^2/\text{sec}$) and 180,000 for the larger component ($s_{20,w} = 7.2S$; $D_{20,w} = 4.64 \times 10^{-7} \text{ cm}^2/\text{sec}$). The values obtained are consistent with the larger component being a complete subunit (two pairs of light and heavy chains) and the smaller component being half of a subunit (one pair of light and heavy chains).

An occasional reoxidation has produced a third component, which sedimented with an $s_{20,w} = 10S$ (Figure 11). Attempts to isolate this component have thus far been unsuccessful. However, in each case where this 10S component could be demonstrated, the reoxidation mixture could precipitate SI in gel diffusion (Figure 8). Thus, it would appear that the minimum size aggregate of γ M-subunits which were capable of cross-linking antigen had an $s_{20,w} = 10S$. Such aggregates could consist of two subunits (four pairs of light and heavy chains) but their precise characterization requires their isolation. The smaller units, $s_{20,w} = 4.9S$ and $7.2S$ have been shown to be incapable of cross-linking antigen molecules but apparently many of them have at least one intact binding site as evidenced by the experimental results described in the next section.

Retention of Antibody Activity by the Reductively Produced Subunits of γ M-antibody. Several reports have appeared in the literature suggesting that at least a portion of the reductively produced subunits of γ M-antibody bind specifically to antigenic sites (Chan and Deutsch, 1960; Jacot-Guillarmod and Isliker, 1962 and 1964; Onoue, et al., 1964), however, the extent of this binding has not been quantitated.

Preliminary investigations using SI labelled with ^{125}I to detect binding of subunits to antigen were carried out using radioimmuno-electrophoresis. This technique depends on the anti-SI subunits being precipitated in the gel by an anti- γ M-globulin which in turn will bind the radiolabelled SI. A normal photograph of the developed and stained slide and a radioautograph of the same slide are shown in Figures 13A and B. Radioautographs prepared with whole anti-SI serum shows that radioactivity was precipitated only in the region in which γ M-antibody would be expected to be found. A concomitant experiment carried out using the same antisera after it had been reduced with 0.1 M mercaptoethanol and then alkylated with iodoacetamide (the well labelled WSr in Figures 13A and B) also showed a band of radioactivity (Figure 13B) only in the position that one finds γ M-subunits precipitated by the anti-immunoglobulin serum. No band of radioactivity could be detected in the absence of the anti-immunoglobulin serum (not shown). The results obtained using isolated γ M-antibody were similar to those described above. In Figure 13C one can see precipitin bands were formed with the γ M-antibody (γ M) and anti- γ M-antibody (A- γ M) ^{125}I - SI (SI*) mixture as



FIGURE 13: Immuno-electrophoretic and radioimmuno-electrophoretic analyses of anti-SI serum and γ M-antibody. The pictures in the left hand column are normal photographs while those in the right column are the corresponding radioautograph. The symbols used indicate: A-Ig, rabbit anti-immunoglobulins; A- γ M, goat anti- γ M-antibody; γ M, γ M-antibody; γ Mr, γ M-antibody reduced with 0.085 M mercaptoethanol and alkylated with iodoacetamide; WS, whole horse antiserum; WSR, horse antiserum reduced with 0.1 M mercaptoethanol; SI*, ^{125}I labelled Type I polysaccharide; +, anode.

well as with SI* alone. However, the γ M-subunits (γ Mr) only formed a band with the A- γ M + SI* mixture (Figure 13, A and B top, C and D bottom). The radioautograph shows that each of the bands had radio-label associated with it and therefore SI, and that there were no additional detectable labelled precipitin bands.

This qualitative demonstration of at least partial retention of active antibody sites following treatment with 0.1 M mercapto-ethanol led to an attempt to quantitate this activity. The method of choice was tube coprecipitation, a procedure in which the reactions that occurred in the agar gel were essentially carried out in a series of tubes. This was accomplished by allowing the antibody preparation to react with a predetermined optimal amount of ^{125}I - SI for one hour at 37°C . An amount of anti-horse immunoglobulin serum that would maximally precipitate the anti-SI was added. The mixture was incubated at 37°C for one hour, then at 2°C overnight. The precipitate was collected, washed, and used for subsequent determinations. The experimental results shown in Figure 14 indicate that, in this system, the amount of non-specific coprecipitation is not large relative to the amount of ^{125}I - SI specifically precipitated. These data were obtained by adding increasing quantities of ^{125}I - SI to a duplicate series of tubes containing intact γ M-antibody. One of the series also received increasing amounts of rabbit anti- γ M-globulin over a range that included amounts of the rabbit antisera equal to or greater than that required to just precipitate all of the γ M-antibody. Following incubation at 37°C for one hour and at 2°C for 18 hours, the precipitates were collected, washed

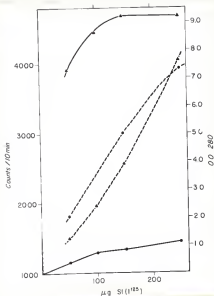


FIGURE 14: ^{125}I - SI precipitated and coprecipitated by anti-SI. The optical density at 280 m μ is shown by the solid line while the counts are shown by the broken line. The corresponding density-count lines use the same symbols. The closer circles (O) representing direct precipitation by anti-SI while the closed triangles (Δ) represent coprecipitation.

several times with cold saline, then dissolved in 0.1 N NaOH. A 0.2 ml portion of the dissolved precipitate was counted in a liquid scintillation counter and the balance was used to determine the total amount of precipitate formed from optical density measurements. As can be seen in Figure 14, although there was, in some cases, as much as an 8.5 fold difference in the amount of protein precipitated in the two series, the number of counts precipitated by antibody in the presence of a given amount of antigen ($^{125}\text{I} - \text{SI}$) were essentially identical whether anti- γM -globulin had been added or not.

Since the above experiment indicated that non-specific precipitation of label was negligible in this system, tube coprecipitation seemed a reliable method to use to quantitate the antibody activity of the subunits.

The data presented in Table 1 are the results of two series of experiments performed on different γM -antibody preparations. It was obvious from these results, that the subunits almost quantitatively retained their ability to bind antigenic determinants of the $^{125}\text{I} - \text{SI}$. The γM -subunits coprecipitated 104% (Experiment 1) and 95.7% (Experiment 2) of the antigen directly precipitated by intact γM -antibody. Under the conditions of the experiment, γG -globulin isolated from a nonimmunized horse also precipitated some counts in the presence of the anti-immunoglobulin. This precipitation of $^{125}\text{I} - \text{SI}$ may be interpreted to mean either or both of two possibilities. The first of these was that the counts found in precipitates formed with normal horse γG -globulin were truly the result of non-specific coprecipitation, in which case the retention of active sites should

TABLE I

Quantitative Co-precipitation of ^{125} I Labeled S I by Anti-Horse Immunoglobulins and γ M-antibodies, γ M-subunits, or Other Horse Immunoglobulins

Horse Globulin Present ^a	Anti-Horse Immunoglobulins Present	Experiment 1		Experiment 2
		Counts/10 min ^b	OD _{280nm} ^c	Counts/10 min
γ M-antibodies	-	5038	0.375	3057
	-	4685		2959
γ M-antibodies		5289	3.94	3619
		4929		3306
γ M-subunit		5098	4.58	2918
		5069		2842
γ G-globulin (normal)		NO ^d		1389
		NO		1393
γ G-globulin (immune) ^d		1843	3.76	NO
		2034		NO
6.4S immunoglobulins		--		4036
		--		3551

^a A 0.5 mg amount of γ M-globulin or γ M-subunits and a 0.8 mg amount of the other immunoglobulins was used to yield the same final amount of precipitate.

^b Net counts after correcting for background (457, exp. 1; 449, exp. 2).

^c Optical density expressed as that of a solution of the precipitate in 1.0 ml of 0.1N NaOH.

^d γ G-globulin isolated from serum of the immunized horse by the same method used to isolate γ G globulin from the unimmunized horse.

^e Not Done.

be quantitated with respect to the counts precipitated by intact γ M-antibody in the presence of the anti-immunoglobulins. Expressed in this manner, the γ M-subunits coprecipitated 99.5% (Experiment 1) and 82.2% (Experiment 2) of the antigen that was coprecipitated by γ M-antibody. The other explanation could be that there were circulating antibodies in the normal serum that cross-reacted with the SI. Polyuronic acids are prevalent in nature, for example pectin and pectic acid, and some of these are known to cross-react with SI (Hill, 1964). If this is the case, then the counts in the coprecipitates of the γ G-globulin, normal and immune, may not be true coprecipitation but may be due to the presence of pre-existing cross-reacting antibody.

Finally, Table 1 shows that the pool containing the '6.4S immunoglobulins' were, on a weight basis, slightly more effective as coprecipitants with antiglobulins than either γ M-antibody or its subunits.

In another series of experiments, agglutination of SI sensitized sheep erythrocytes was used as a sensitive indicator for cross-linking in order to assay for multiple antibody sites, on a single subunit of γ M-antibody. Using intact γ M-antibody, as little as 0.53 μ g/ml of antibody could be detected. No agglutination could be detected using the 4.9S or 7.1S subunit preparations, even at concentrations of about 3,000 μ g/ml. However, the addition of a small amount of a rabbit anti-horse immunoglobulin serum to the reaction mixture produced agglutination in those tubes in which the concentration of subunit was relatively high, i.e. over 0.5 μ g/ml.

The quantitative retention of antibody active sites by the 7.1S subunit was demonstrated using the Farr technique. In the Farr technique, the soluble antigen-antibody complexes are precipitated at 50% saturation with ammonium sulfate (Farr, 1958). The results of this experiment are shown in Table 2. At the maximum precipitation of radioactivity by the subunit, obtained with 0.2 ml of ^{125}I - SI, the subunit bound 96% of the radioactivity which was bound by intact γM -antibody indicating that the 7.1S subunits quantitatively retained their ability to bind antigenic determinants.

To carry out the experiments described in the preceeding section, it was necessary to prepare a radiolabelled polysaccharide. Since the method used for this purpose was a novel one and has not been described in detail, this section is concerned with the preparation of extrinsically labelled polysaccharides.

Characterization of the ^{125}I labelled SI. The need for a radio-labelled SI could be satisfied by one of two general methods. The SI could be labelled in vivo by feeding the growing bacteria a radioactive precursor which would be incorporated, at least in part, into the polysaccharide capsule. This method requires a relatively large amount of the label as part of it would be incorporated into other cell components or products. Once the radioactive polysaccharide was laid down, it would be necessary to isolate the SI as previously described. Another method would be to extrinsically label the SI. Technically, this method was preferred as it involves a minimum number of manipulations of radioactive substances.

A method of extrinsically labelling the SI with radioiodine was suggested in the introduction to a paper by Landauer and Rydon (1953).

TABLE 2

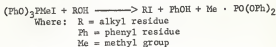
Ability of γ M-subunits to Bind SI
as Detected by the Farr Method ^a

Antibody	Vol ^{125}I - SI	Counts/10 min ^b in precipitate
γ M	0 ml	0
0.75 μ g	0.05 ml	852
	0.10 ml	1598
	0.20 ml	2389
	0.30 ml	2506
7.1S subunit	0 ml	0
0.75 mg	0.05 ml	800
	0.10 ml	1298
	0.20 ml	2293
	0.30 ml	1778

a) In the Farr method (1958) the antigen is incubated with the antibody preparation, then the antibody is precipitated with ammonium sulfate. In this case the antibody was precipitated at 50% saturation with the salt. The above counts have been corrected for a small amount of non-specific precipitation of the ^{125}I - SI.

b) Corrected for background counts.

In this paper it was mentioned that alkyl halides could be prepared by the reaction of triphenyl phosphate methiodide with an alcohol according to the following reaction:



This reaction was found to be adaptable to iodinating carbohydrates using the procedure described in the Materials and Methods section.

In an early attempt to assure ourselves that the ^{125}I was covalently bound to the carbohydrate, dextran, of average molecular weight of 153,000, was reacted according to the described procedure and then was passed through a 2.5 x 40 cm column of Bio-Gel P-30 (Bio-Rad Labs., Richmond, Calif.). Figure 15 shows that, by far, the majority of the radioactivity (bar graph) was associated with dextran (line graph). There remained a small amount of free radioiodine which was retarded on the column and eluted after the dextran.

In all preparations of SI there was a small amount of contaminant that adsorbed at 280 mμ. To exclude the possibility of the radioiodine being associated solely with this material, the ^{125}I - SI was subjected, in turn, to digestion with RNase, DNase, and pronase, at the optimal pH for each enzyme, with concomitant dialysis against buffer at the appropriate pH. Following each treatment there was a small reduction in specific activity which could be accounted for by the appearance of radiolabel in the dialysate. Most of the

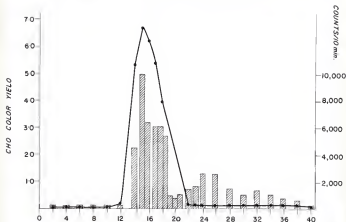


FIGURE 15: Gel filtration of ^{125}I - Dextran on Bio-Gel P-30. The concomitant elution of the majority of the ^{125}I (bar graph) with the dextran (line graph) indicates that the ^{125}I was bound to the dextran. Carbohydrate was determined in the effluent by the orcinol method and is expressed as $10 \times$ optical density at 540 m μ . Counts were determined for 1 ml samples of each fraction.

280 mμ absorption remaining was that expected from the concentration of pronase used for digestion. This enzyme treated ^{125}I - SI was then passed through a 2.5 x 90 cm column of Bio-Gel P-30.. In Figure 16 it can be seen that the uronic acid curve and the radioactivity curve are almost superimposable while the 280 mμ absorbing material eluted after a larger volume of water was passed through the column.

The retention of antigenic integrity by the ^{125}I - SI is shown in Figure 17. These data were obtained by adding increasing amounts of γM -antibody to a constant amount of the ^{125}I - SI. The precipitate was washed with cold 0.9% saline and dissolved in 1 ml of hyamine. The precipitate and the first supernatant, normalized to 1 ml, was counted in the scintillation counter. It can be seen in this figure, that at maximum precipitation, at least 70% of the total counts were precipitated by the anti-SI (shown by open squares in Figure 17). The radioactivity that remained in the supernatant after precipitation with the anti-SI is shown by the closed circles and it amounts to only 20% of the total radioactivity added to the mixture. The open triangles are the sum of the counts precipitated plus those remaining in the supernate at each point. The decrease noted here between the sum and the total activity added is most probably due to quenching by the protein present in the antibody precipitated label. The labelling reaction requires rather long exposures of the polysaccharide to temperatures above ambient and during this time there may be some loss of antigenic reactivity which could account for the non-precipitated counts.

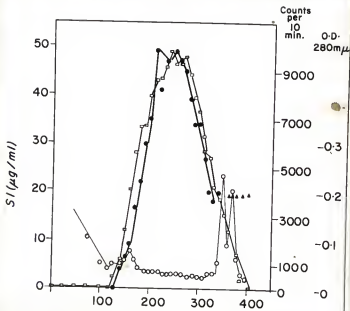


FIGURE 16: Bio-Gel P-300 chromatography of ^{125}I - SI. The SI elution is shown by the closed circles (●); radioactivity by open rectangles (□) and optical density at 280 mμ by open circles (○). The fractions represented by the closed triangles (▲) gave a brown color in the uronic acid determination due to the protein present.

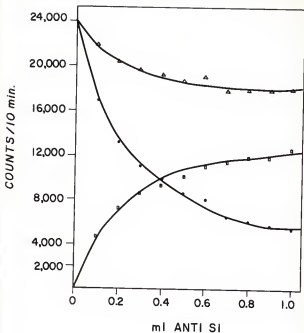


FIGURE 17: Integrity of the antigenic determinants of SI following labelling with ^{125}I . The radioactivity precipitated by the indicated amounts of anti-SI are indicated by the open rectangles (), that remaining in the supernatant by the closed circles (), and the sum of the two at each point by the open triangles ().

Hence, from the data found, it would appear that a significant portion of the radioiodine is covalently bound to the polysaccharide. In the case of dextran, it was found that the technique resulted in conjugation of 80 moles of iodine per mole of dextran (mw = 153,000) or, one iodine atom per 10 glucose residues. Evidence seems to indicate that the number of moles of iodine reacting with a mole of SI is about the same. The molecular weight of SI is 171,000, or slightly more than the dextran, while the number of counts per unit time per mg are also slightly higher. Labelling with this number of molecules of iodine was sufficient for the proposed use but did not result in an extensive loss of antigenic specificity. This technique has been successfully employed in another laboratory to radio-label human erythrocyte 'A' substance. Thus, it would seem that the basic technique, with slight modifications, will be useful in extrinsically labelling carbohydrates in general.

Biologic Activity of Anti-SI Antibodies. The exquisite sensitivity of mice to fatal infections with pneumococci provides a sensitive system for examining the in vivo activity of various antibody preparations. The protective activity of antisera is directly related to its content of antibodies to the polysaccharide of the capsule.

The anti-SI serum, diluted to a final concentration of 0.001 $\mu\text{g/ml}$ of antibody as determined by quantitative precipitin curves, protected 50% of the mice to a challenge dose of 100 pneumococci. As little as 0.0001 $\mu\text{g/ml}$ of purified γM -antibody would confer the same degree of protection. Thus, the purified antibody was ten times

more effective in protecting the mice. This apparent increase in the ability to prevent a lethal infection in mice upon isolation of the γ M-antibody was probably due to removal of the other, less efficient, antibodies from competition for antigenic sites. In contrast, 10 μ g/ml of γ G-antibody or 0.1 μ g/ml of γ A-antibody were required to protect 50% of the mice.

Reduced serum or reduced γ M-antibody did not protect the mice at concentrations up to 10 μ g/ml. The 7.1S subunit of γ M-antibody, which was shown to render the bacteria agglutinable by an anti-immunoglobulin serum, conferred no protection when tested at 100 or 1,000 μ g/ml. The results of experiments yielding the above data are shown in Table 3/.

TABLE 3

Protection Against Type I Pneumococcus Infection in the
Mouse by Equine Anti-SI Immunoglobulins

Immunoglobulin	Concentration	Survival
Whole Antiserum	10 to 0.01 $\mu\text{g/ml}^a$	26/26
	0.001 $\mu\text{g/ml}$	5/9
Reduced Antiserum ^b	10 to 0.1 $\mu\text{g/ml}^a$	0/15
γM -Antibody	10 to 0.001 $\mu\text{g/ml}$	39/40
	0.0001 $\mu\text{g/ml}$	2/5
Reduced γM -Antibody ^b	1000 to 100 $\mu\text{g/ml}$	10/10
	10 to 0.1 $\mu\text{g/ml}$	0/25
γG -Antibody	1000 and 100 $\mu\text{g/ml}$	10/10
	10 $\mu\text{g/ml}$	6/9
	1.0 $\mu\text{g/ml}$	0/9
γA -Antibody	100 and 10 $\mu\text{g/ml}$	15/15
	1.0 $\mu\text{g/ml}$	9/10
	0.1 $\mu\text{g/ml}$	5/9

a) Concentration of antibody based on quantitative precipitin curves on whole serum.

b) Reduced at a final concentration of 0.1 M 2-mercaptoethanol.

DISCUSSION

Investigations of the physicochemical properties of horse γ M-antibody undertaken in this study show that: The γ M-antibody possess antigenic sites that are unique to the γ M-globulins as well as antigenic sites that are shared with the other class of immunoglobulins. The intact molecule was depolymerized to subunits by reduction of disulfide bonds with various thiol reagents, however while certain secondary activities were lost, the subunits of certain sizes retained the ability to specifically bind antigen. Reoxidation of the disulfide bonds of γ M-subunit gave circumstantial evidence that a combination of subunits having an $s_{20,w}$ of about 10S was the minimum size that would cross-link antigen molecules and lastly, γ M-antibody was found to be 10^5 times more effective than γ G-antibody and 10^3 times more effective than γ A-antibody in protecting mice from a lethal infection when challenged with an injection of 100 lethal doses of Type I pneumococci.

Purified horse macroglobulin antibody was employed in the initial studies which defined γ M-globulin (γ_1 M-globulin) as an immunoglobulin (Heidelberger and Pedersen, 1937; Kabat and Pedersen, 1938) and which distinguished this protein from γ G-globulin on the basis of its sedimentation properties and molecular weight (Kabat, 1939). In the intervening years, most of the comparisons of the antigenic and physicochemical properties of γ M-globulin with those of γ G-globulin

have utilized human γ M-immune globulin, normal γ M-globulin, Waldenström's macroglobulin, γ M-globulin that was rich in rheumatoid factor, or whole serum. In this present study specifically purified horse γ M-antibody directed against the defined antigen, SI, has been again employed. Many of the relationships between γ M- and γ G-globulins and certain characteristics of γ M-globulin that were found for the horse immunoglobulins paralleled or were similar to those previously found for the corresponding human globulins. Thus, purified horse γ M-antibody was found to contain unique antigenic sites which permitted its differentiation from γ -globulins of other classes (γ A, γ G). The class specificity of human γ M-globulin has been shown by Franklin and Kunkel (1955), who used a crude normal γ M-globulin preparation, and by Korngold and van Leeuwen (1957), who used Waldenström's macroglobulin. Also, just as human or rabbit γ M- and γ G-globulins have been found to be antigenically related (Fahey, 1963; Cebra and Goldstein, 1965) due in part to the presence of similar light chains in both molecules, the horse γ M-antibody was found to be antigenically related to γ G-globulin. Light chains prepared from both horse γ G- and γ M-globulins gave reactions of identity when tested with rabbit anti-horse γ G-globulin. Light chains prepared from both horse γ G- and γ M-globulins gave reactions of identity when tested with rabbit anti-horse γ G-light chain sera. Chaplin, et al. (1965), on the other hand, have found close agreement between the amino acid compositions of the light chains derived from horse γ G- and γ A-globulin, so it appears that all three horse immunoglobulins (γ G-, γ A-, and γ M-) will be found to contain the same kinds of light chains, as is the case in the human system.

Using a series of pathologic human macroglobulins, Deutsch and Morton (1957, 1958), first showed that γ M-globulins were susceptible to reductive depolymerization with 0.1 M mercaptoethanol. A similar reduction of horse γ M-antibody to subunits was found to occur on incubation for 30 minutes with concentrations of mercaptoethanol 0.075 M and higher. Incubation in mercaptoethanol at concentrations of 0.04 M to 0.06 M resulted in depolymerization of only a portion of the molecules. Another reducing agent, dithiothreitol, was found to depolymerize reductively γ M-antibody at much lower concentrations than that required for mercaptoethanol. The increased efficiency of dithiothreitol can be easily shown to be a result of the nature of the molecule. Each molecule contains two sulfhydryl groups separated by four carbon atoms. The two sulfhydryl groups are necessary to reduce completely both members of the broken disulfide and the bond angles of the DTT molecule are such that a stable, five atom ring structure can be formed. Horse γ M-antibody is completely reduced to subunits by as little as 0.005 M dithiothreitol at pH 6.8 or 0.002 M at pH 8.5.

The concentration of mercaptoethanol required to dissociate the horse γ M-antibody is considerably higher than that required to halve the '5S product' of pepsin digestion of rabbit γ G-globulin (0.008 M mercaptoethanol, pH 5, one hour at 37°C) (Mandy and Nisonoff, 1963). In fact, the concentration of a monothiol reducing agent (0.075 to 0.1 M) required to depolymerize γ M-globulin was very close to that (0.06 M) found by Fleishman, *et al.* (1963) to be sufficient to reduce all of the interchain disulfide bonds in horse γ G-globulin.

Thus, it appears that reductive depolymerization of macroglobulin antibody requires cleavage of very resistant or very many disulfide bonds. Indeed, Jacot-Guillarmod and Isliker (1962) found that about 22 disulfide bonds were cleaved on depolymerization of human γ M-globulin with 0.01 M borohydride and, in fact, this is the same number as we found to be cleaved in horse γ M-antibody following reduction with 0.0025 M dithiothreitol. One might anticipate that some of the light chains of γ M-globulin would no longer be covalently bound to the rest of the molecule after its depolymerization. Starch gel electrophoresis of reduced horse γ M-antibody under conditions known to dissociate the chains of reduced γ G-globulin, pH 3.0, 8 M urea, (Edelman and Poulik, 1961) did reveal the presence of a second, rapidly migrating component in the subunit mixture, which could have been from light chain (Hill, 1964). Free light chain was then isolated by dissociating it from the subunit in 0.5 M propionic acid, 6M in urea and subsequent gel filtration.

Reduction of the horse γ M-antibody to subunits resulted in loss of its ability to precipitate antigen in solution or in agar gel. Through the years, the loss of a variety of activities indicative of antigen-antibody interaction upon reduction of whole serum or immune globulin has been taken to demonstrate the macroglobulin nature of the antibody under study. However, that loss of precipitating or agglutinating ability upon reduction need not indicate inactivation of the antibody combining sites of γ M-globulin was suggested by the finding of Chan and Deutsch (1960). These authors

showed that reduced human Rh saline agglutinins of the γ M-type still specifically associated with the surface antigens of the appropriate erythrocytes although they no longer agglutinated the cells. The erythrocytes coated with γ M-subunits could then be agglutinated by a rabbit anti-human macroglobulin serum. Recently, Schrohenloher, et al. (1964) have found that subunits obtained by reduction of human macroglobulin, which was rich in a pathologic anti-human γ G-globulin (rheumatoid factor) still associated with the human globulin antigen to form soluble complexes. Onoue, et al. (1964) used yet another technique, radioimmuno-electrophoresis (Hochwald, et al., 1961), to demonstrate the binding of ^{131}I - labelled p-azobenzenearsonate-insulin conjugate by the subunits of rabbit γ M-antibody prepared by reduction of a purified antibody preparation containing 39% γ M-globulin. A specific sheep anti-rabbit γ M-globulin was used to coprecipitate the labelled antigen in agar gel along with the subunits. Using radioimmuno-electrophoresis we confirmed the retention of binding activity by the γ M-subunits that were present in both reduced anti-SI sera or that were derived from purified, homogeneous γ M-antibody by using ^{125}I - SI and a goat anti-horse γ M-globulin for coprecipitation. To quantitate the amount of antigen binding activity retained by the γ M-subunits, relative to their parent intact γ M-antibody, quantitative tube coprecipitation was used. There was no significant difference between the amount of ^{125}I - SI coprecipitated with anti- γ M-globulin by the intact γ M-antibody or by the γ M-subunit. Thus, our results indicate that reductive depolymerization of purified horse γ M-antibody does not appreciably inactivate antibody combining sites.

The integrity of a particular tertiary structure of a protein is usually a requirement for its activity. Proteins, which have some functional activity which can be measured, often show a great alteration in activity, either qualitatively or quantitatively, when changes in their tertiary structure are induced. In addition, certain complex proteins, composed of several polypeptide chains or subunits, shown an alteration in their functional activity upon suffering changes in their quaternary structure. For example, enzymes whose quaternary structures are stabilized by electrostatic as opposed to covalent bonds, can be dissociated and reassociated freely. Glutamic dehydrogenase can be dissociated into several subunits that no longer have glutamic dehydrogenase activity but which now have alanine dehydrogenase activity (Tomkins and Yielding, 1961). This conversion is freely reversible and, in fact, seems to be utilized as a control mechanism for a particular reaction.

Restoration of the original configuration and activity of a protein is usually more difficult if the depolymerization involves breaking covalent bonds. The covalent bond almost exclusively involved in the stabilization of the quaternary structure of proteins is the disulfide bond. Disulfide bonds may be formed between cysteine residues on the same polypeptide chain (intrachain) or between residues on different polypeptide chains (interchain). With rare exceptions, one is unable to break selectively one particular bond within one of these groups and not the others. Protein molecules often contain several intrachain as well as several interchain disulfide bonds. The selectivity one can achieve in the

reduction of these disulfide bonds leading to depolymerization of a protein will be dependent on the folding of the chains and their propensity, or lack of it, to remain associated by non-covalent forces. The interchain disulfide bonds of γ G-globulin are readily accessible to reduction by thiols, complete reduction being achieved with as little as 0.75 M thiol. However, there is little or no detectable dissociation of the various polypeptide chains or combinations of polypeptide chains that are known to make up the molecule. Thus, the quaternary integrity of γ G-globulin remains intact until one brings about unfolding of the molecule by interference with its non-covalent bonds. Reagents commonly employed are 1 M propionic acid, 6 M urea, or 6 M to 8 M guanidine. The propensity of the polypeptide chains of γ G-globulin to remain associated contrasts with an apparent lack of non-covalent association of the subunits of γ M-globulin. One need only to reduce a requisite number of interchain disulfide bonds to achieve dissociation of the molecule into its subunits.

Reducing agents used at concentrations which just lead to complete depolymerization of γ M-globulin do not selectively reduce only those interchain disulfide bonds holding the subunits together but also reduce a significant number of disulfide bonds between the μ -polypeptide chain and the light polypeptide, which remain associated as do the heavy and light chains of γ G-globulin. Upon subsequent treatment and chromatography in the presence of propionic acid and urea, 32% of the total light chains can be shown to be no longer covalently bound to μ -chains. The location of the disulfide bonds

responsible for the linking of the subunits in the γ M-globulin molecule, which can be between light-light, μ - μ , or light- μ polypeptide chains, has not been elucidated.

Reoxidation of reduced intrachain disulfide bonds under conditions that favor disulfide interchange, will lead to reformation of most of the original disulfide bonds. Of course, those disulfide pairs which did not become grossly disorientated will be correctly reoxidized early on while other pairs form at random. The final disulfide bonding achieved will be that results in the most thermodynamically stable configuration under that condition. The disulfide bonds in native protein represent the most stable configuration under physiologic conditions (Givol, *et al.*, 1965). When more than one polypeptide chain is involved, reoxidation to an active configuration depends on the proper interchain disulfide bonds being reformed as well as the intrachain disulfide bonds of the polypeptide chains. Again, if the chains remain associated by non-covalent bonds, it is relatively easy to have the same sulphydryl residues reoxidize and form a disulfide bond. If the chains become even partially dissociated or rearranged, the reformation of the proper disulfide bond become less probable.

Reoxidation of the subunits of horse γ M-globulin fit the latter category. Reduction of the molecules leads to a complete dissociation of the 7.1S subunits and the production of a large number (21 to 44 depending on the concentration of thiol) of free sulphydryl residues. Assuming that the 44 free sulphydryl groups are about equally distributed among the five subunits, then there are about

nine sulfhydryl groups in each subunit. At least a portion of these remain sterically close to each other due to non-covalent forces, hence, are able to readily reform, however, this still allows for a large number of possible intra-subunit combinations of disulfide bonds if all are sterically possible in addition to the inter-subunit disulfide bonds. Most likely the probability of formation of an intra-subunit disulfide bond upon reoxidation is much higher than the chance of formation of a link between two subunits and this makes reaggregation of two subunits difficult. Formation of the correct disulfide bond within the cell synthesizing γ M-antibody is facilitated by an innate propensity to noncovalently associate, thereby aligning the sulfhydryl groups that will subsequently covalently link the subunits. In vitro the subunits prepared by the described method do not appear to exhibit this propensity. Either the concentrations of the subunits achieved in the reoxidation mixtures are too low to result in close packing of the molecules so that the electrostatic forces can become effective, or disruption of large numbers of disulfide bonds upon reductive depolymerization results in modifications of the tertiary structure of the subunits and a concomitant interference with complementary electrostatic bonding.

The aggregates that are formed by reoxidation are able to express at least some of their former biologic activity, for example, the precipitating of SI or agglutinating of sensitized erythrocytes. Restoration of the ability to produce some of the secondary effects of γ M-antibody that are due to its cross-linking properties by

oxidative recombination of its subunits has been reported by Jacot-Guillarmod and Isliker (1962). These workers succeeded in restoring the agglutination titer of reduced human anti-A or anti-B γ M-iso-antibodies by immediate dialysis of the reduction mixture against Veronal buffer, pH 8.4, according to the procedures which were used by Deutsch and Morton (1958) oxidatively to recombine subunits of Waldenström's macroglobulin. Likewise, Schrohenloher, et al., (1964) have partially recombined the subunits of rheumatoid factor in the same manner and observed that the reoxidized preparations regained the ability to precipitate with human γ -globulin. Removal of thioglycolate from depolymerized horse γ M-antibody on Dowex-1-acetate, followed by pressure dialysis against phosphate-borate buffer, pH 8.0 or depolymerization with 0.0025 M DTT followed by dialysis against phosphate-borate buffer, pH 8.4, leads to partial reassociation of subunits and restoration of precipitating ability. Thus, it seems that γ M-subunits retain active antibody sites but that they are unable to cross-link particles or antigen. Even partial reassociation of the subunits restores significant precipitating ability. The subunits presumably cannot cross-link antigen because either they are univalent or because their sites are so oriented that they cannot combine with more than one high molecular weight antigen at a time.

Subunits of the above type that are reaggregated in a fashion that permits them to express a secondary biologic activity may be artifacts and may bear no resemblance to the native molecule. Intermediate polymers of the 7.1S subunit, between one (the subunit) and

five (the intact γ M-globulin) are not found by reductive depolymerization but only by reoxidation of the subunits. In connection with this, one wonders why it is necessary to break so many disulfide bonds to depolymerize the γ M-globulin. I feel that, in reality, there are relatively few easily reduced disulfide bonds holding the subunits together. Before the molecule can dissociate, sufficient disulfide bonds, other than those holding the subunits together, must be broken in order to modify the secondary and tertiary structure. There is circumstantial but reasonable evidence to support the above observations. If a large number of disulfide bonds do hold the subunits together and all that is necessary to depolymerize the molecule is to break these, then using limiting levels of reducing agents one would expect random assortments of subunits combinations could be found. As mentioned earlier, this is not the case.

Although the bulk of the antibodies (82%) precipitated from the horse anti-SI serum by the polysaccharide preparation were of the γ M-class, γ G- and γ A-antibodies were also identified. The formation of antibodies of three classes (γ G-, γ M-, and γ A) to a single antigen has been described for the human (Fahey and Goodman, 1964) and the rabbit (Onoue, et al., 1964). Recently Rockey, et al. (1964) have described the presence of a new 10S γ_1 -antibody, as well as γ A-, γ M-, and three distinct γ G-antibodies to the p-azophenyl- β -lactoside group in the horse. In our own anti-SI system, the actual specificities of the γ A-, and γ G-, and γ M-antibodies have not been defined as narrowly. However, it appeared that at least a part of the γ G- and γ A-antibodies reacted with the same polysaccharide molecule that was precipitated by the γ M-antibody.

Since relatively large amounts of these separate classes of antibody are available, it is possible to compare certain of their biologic properties such as their opsonizing and complement fixing activities. Some preliminary attempts to show complement fixation by the anti-SI antibodies have not met with success. γ M-antibody from other animals are known to efficiently fix complement, for example, rabbit anti-sheep erythrocytes. Guinea pig serum is the usual source of hemolytic complement and also served as the source reported above. Thus, one can say that horse anti-pneumococcal antibody does not fix guinea pig complement. In our case, we also used guinea pig serum as a complement source. That the participation of guinea pig complement in an antigen-antibody reaction that takes place in horse antiserum may be blocked by components of the horse's own complement system has been suggested by Nelson (1965). Using purified horse γ M-, γ G-, and γ A-anti-SI we could not find any hemolytic complement activity with SI conjugated sheep erythrocytes. Thus, it appears that guinea pig complement is not compatible with the antibodies of the horse. The role of horse complement has not been investigated.

In the horse anti-SI system, as well as in the S. typhimurium system of Robbins, et al. (1965), γ M-antibody was found to have a much higher in vivo opsonic activity in the mouse than either γ G- or γ A-antibody. In vivo protection of 50% of the mice challenged with 100 pneumococci, about 100 L.D. 50's, required 10^5 times more γ G-antibody than γ M-antibody. The γ A-antibody was also less effective

than γM -antibody, 10^4 times more γA -antibody than γM -antibody being required to achieve the same degree of protection. The protection conferred by antibodies to pneumococci is by virtue of the bacteria becoming very susceptible to phagocytosis. The mere coating of antibody onto the capsular surface does not seem to explain 'opsonization' in this case. The γG -antibodies have antibody activity directed against the polysaccharide and solutions of equal concentration would contain approximately five times as many molecules of γG - as γM -antibody. Also, a given solution of γM -antibody, upon reduction would again have five times as many units capable of binding to the surface of the pneumococci, yet, in both of these cases relatively large amounts of the smaller proteins are required. Thus, the explanation for its greater effectiveness must lie in the integrity of the γM -molecule or its unique quaternary structure.

The physical structure of horse γM -globulin has not been elucidated. From what is known, many structures can be postulated. The molecule is made up of five covalently linked subunits, each consisting of two light chains and two heavy chains, analogous to γG -globulins; yet, no more than five antibody sites have been detected in the intact molecule and consequently only a single site on each subunit is apparent. The γM -globulin molecule is only slightly wider than γG -globulin, but much longer; $47A^\circ \times 946A^\circ$ for horse γM -globulin and $39A^\circ \times 286A^\circ$ for horse γG -globulin (Campbell and Bullman, 1952). If these dimensions reflect a more or less stable configuration for the molecule then the spatial arrangement of the subunits in an intact γM -globulin molecule is not easily visualized. The

subunits have a molecular weight somewhat higher than γ G-globulin, hence, a slightly larger size. They are more asymmetrical than γ G-globulin, but not as asymmetric as γ M-globulin itself. The asymmetry has been ascertained from the frictional ratios found for the human to be 1.92, 1.69, and 1.50 for γ M-, γ M-subunit, and γ G-globulin respectively (Miller and Metzger, 1965). The problem of building a model of γ M-globulin then depends on how one may covalently bind five subunits into a unit that is less than 1.25 times as wide and 3 times as long as its individual subunit.

Table 4 summarizes the findings of the author for horse γ M-antibody, γ M-subunit, γ G-antibody, and γ A-antibody. Table 5, as a comparison, shows the findings of others for various immunoglobulins.

TABLE 4
A Comparison of the Properties Found for Horse Immunoglobulins
in this Study

Type of Antibody	Antibody Activity	Sedimentation Coefficient	Molecular Weight	Effect of Thiols
γ M-antibody	precipitating and agglutinating	17.3S	900,000	depolymerization
γ M-subunit				
4.9S	some binding	4.9S	100,000	----
6.5S	quantitative binding	6.5S	---	----
7.1S	quantitative binding	7.1S	180,000	----
9.8S	possible precipitating and agglutinating	9.8S	---	----
γ G-antibody	precipitating and agglutinating	6.4S	---	no effect
γ A-antibody	binding precipitating?	10.0S	---	----

TABLE 5
Properties of Immunoglobulins from Various Species

Species and Globulin	Antibody Activity or Serum Source	Sedimentation Coefficient	Molecular Weight		Reference
			Intact Molecule	Heavy Chain	
<u>Human</u>					
Waldenström's	pathologic	17.90S	890,000	---	Miller and Metzger, 1965
Waldenström's subunit	pathologic	7.07S	185,000	---	ibid
γG-globulin	normal	6.71S	160,000	---	ibid
<u>Rabbit</u>					
γM-globulin	anti-S. <u>enteritidis</u>	---	900,000	70,000	Lamm and Small, 1966
γM-subunit	anti-S. <u>enteritidis</u>	---	180,000	---	Lamm and Small, 1966
γG-globulin	normal	---	140,000	53,000	Small and Lamm, 1966
<u>Swine</u>					
γM-globulin	anti-pneumococcus	18.00S	930,000	---	Kabat and Pedersen, 1938 Kabat, 1939
<u>Equine</u>					
γM-globulin	anti-pneumococcus	19.30S	920,000	---	Kabat and Pedersen, 1938 Kabat, 1939
γG-globulin	anti-diphtheria toxoid	7.20S	184,000	---	Pappenheimer, <u>et al.</u> , 1940

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This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Medicine and to the Graduate Council, and was approved as partial fulfillment of the requirements of the degree of Doctor of Philosophy.

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